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Title page

Preclinical pharmacology of [2-(3-fluoro-5-methanesulfonylphenoxy)ethyl](propyl)amine (IRL790), a novel dopamine transmission modulator for the treatment of motor and psychiatric complications in Parkinson's disease

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Running title: Pharmacology of IRL790, novel DA modulator for PD

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14 figures

3 tables

56 References

Abstract : 250 words

Introduction: 741 words

Discussion: 1500 words

Non standard abbreviations:

ACh Acetyl choline

AIMs Abnormal involuntary movements

Arc *Activity regulated cytoskeleton associated protein*

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| | |
|-------------|---|
| DA | Dopamine |
| DOPAC | 3,4-dihydroxyphenylalanine |
| <i>Egr1</i> | <i>Early growth response protein 1</i> |
| HPLC/EC | High performance liquid chromatography/electrical detection |
| 5-HIAA | 5-hydroxyindoleacetic acid |
| 5-HT | 5-hydroxytryptamine |
| HVA | homovanillic acid |
| HPRT | hypoxanthine phosphoribosyltransferase |
| IEG | Immediate early gene |
| IRL790 | ([2-(3-fluoro-5-methanesulfonylphenoxy)ethyl](propyl)amine) |
| LIDs | L-DOPA induced dyskinesias |
| MFB | medial forebrain bundle |
| 3-MT | 3-methoxytyramine |
| NA | Noradrenaline |
| NM | normetanephrine |
| NMDA | N-methyl-D-aspartate |
| NPAS | neuronal PAS domain protein |
| Nptx2 | pentraxin 2 |
| PCR | Polymerase chain reaction |
| PD | Parkinson's disease |
| Pfc | Prefrontal cortex |

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PLS Partial least squares

RT Reverse transcription

Recommended section: Drug discovery and translational medicine

Abstract

IRL790, ([2-(3-fluoro-5-methanesulfonylphenoxy)ethyl](propyl)amine), is a novel compound, in development for the clinical management of motor and psychiatric disabilities in Parkinson's disease. The discovery of IRL790 was made applying a systems pharmacology approach, based on *in vivo* response profiling. The chemical design idea was to develop a new type of DA D3/D2 receptor type antagonist built on agonist rather than antagonist structural motifs. We hypothesized that such a dopamine antagonist with physicochemical properties similar to agonists would exert antidyskinetic and antipsychotic effects in states of dysregulated dopaminergic signaling, while having little negative impact on physiological dopamine transmission and, hence, minimal liability for side effects related to dopamine dependent functions. At the level of *in vivo* pharmacology, IRL790 displays balancing effects on aberrant motor phenotypes, reducing LIDs in the rodent 6-OHDA lesion model, and reducing psychostimulant induced locomotor hyperactivity elicited by pretreatment with either d-amphetamine or dizocilpine, without negatively impacting normal motor performance. Thus, IRL790 has the ability to normalize the behavioural phenotype in hyperdopaminergic as well as hypoglutamatergic states. Neurochemical and immediate early gene (IEG) response profiles suggest modulation of DA neurotransmission, with some features such as increased DA metabolites and extracellular DA shared by atypical antipsychotics, and others such as increased frontal cortex IEGs, unique to IRL790. IRL790 also increases extracellular levels of Ach in the prefrontal cortex and ventral hippocampus. At the receptor level, IRL790 appears to act as a preferential DA D3 receptor antagonist. Computational docking studies support preferential affinity at D3 receptors, with an agonist like binding mode.

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Significance statement:

This paper reports preclinical pharmacology, along with molecular modelling results on IRL790, a novel compound in clinical development for the treatment of motor and psychiatric complications in advanced PD. IRL790 is active in models of perturbed dopaminergic and glutamatergic signaling, including rodent 6-OHDA LIDs, and psychostimulant induced hyperactivity, in a dose range that does not impair normal behavior. This effect profile is attributed to interactions at DA D2/D3 receptors, with a 6-8 fold preference for the D3 subtype.

Introduction

The key pathophysiology of PD involves loss of dopaminergic and noradrenergic neurons, in the substantia nigra and locus coeruleus, respectively, associated with severe motor symptoms and progressive autonomic and neurocognitive dysfunctions (Kalia and Lang, 2015; Vermeiren and De Deyn, 2017). Other subcortical, cortical, and autonomic pathologies contribute to non-motor symptoms in PD. It has been suggested that cortico-striatal dysconnectivity and plasticity are key drivers for both core symptoms of PD and adverse effects emerging with long term dopaminergic treatment (Villalba and Smith, 2018).

Involuntary movements occurring on levodopa treatment (LIDs) remain an unmet clinical need since the introduction of levodopa in the 1970s. An estimated 40% of PD patients develop LIDs within 4-6 years of L-DOPA treatment, and over time it afflicts almost all patients (Ahlskog and Muentner, 2001). The pathogenetic mechanisms underlying the development of motor complications emerging with long term L-DOPA treatment are not fully clarified. One major hypothesis is the impact of non-physiological fluctuations in striatal dopamine release, leading to maladaptive changes in dopaminergic and glutamatergic transmission pre- and postsynaptically (Cenci, 2014). Much focus has been on the impact of chronic L-DOPA treatment, but it has also been suggested that dopamine denervation *per se* is the central cause of LIDs (Borgkvist et al., 2018). Both D1 and D2/D3 receptor signaling have been implied in this context.

The development of LIDs is strongly associated with augmented DA D1 receptor mediated neurotransmission, in medium spiny neurons of the direct striatonigral pathway (Feyder et al., 2011). Upregulation of DA D3 receptors has been pointed out as a key factor contributing to dysregulated D1 receptor mediated signaling, through D1–D3 receptor-receptor interactions including heteromerization interfering with D1 receptor internalization and intracellular signaling (Marcellino et al., 2008).

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Striatal dopamine D3 receptors are upregulated in 6-OHDA lesioned rats treated with L-DOPA (Bordet et al., 1997), and in MPTP-treated primates (Bezard et al., 2003). Furthermore, increased DA D3 receptor binding in PD patients with LIDs has been demonstrated using positron emission tomography (PET) (Payer et al., 2016). D3 receptor knock-out reduces LIDs in mice (Solis et al., 2017), while L-DOPA treatment is reported to induce involuntary movements in non-parkinsonian rodents overexpressing D3 receptors (Cote et al., 2014).

In clinical practice, the main strategy to reduce L-DOPA induced motor complications in PD is to reduce, and adjust dopaminergic treatment, to minimize fluctuations in plasma concentrations of the dopamine agonist used (Rascol et al., 2015). This can improve motor fluctuations but does not specifically address dyskinesias. Amantadine was recently introduced in an extended release formulation for treatment of LIDs (Sharma et al., 2018). Among the side effects, psychotic symptoms are likely related to the NMDA antagonist properties of amantadine, and psychotic symptoms are frequently observed in clinical studies on NMDA antagonists (Muir and Lees, 1995). An increased incidence of visual hallucinations was reported in a meta-analysis assessing mGluR5 antagonists in LIDs (Wang et al., 2018b). There are no other pharmacological treatments approved for LIDs. Surgical procedures, including deep brain stimulation, are used in some cases to alleviate dyskinesias and improve overall motor function in PD (Krack et al., 2017).

IRL790 (Sonesson et al., 2012) is a novel compound in development for the treatment of mental and motor complications in PD. The discovery of IRL790 was made applying an *in vivo* systems pharmacology approach (Waters et al., 2017). The strategy was to find compounds that could normalize aberrant phenotypes linked to cortico-striatal dysconnectivity, through interactions with DA D2/D3 receptors. The original chemical design idea was to develop a new type of DA D2/D3 antagonist build on agonist rather than antagonist structural motifs. This would lead to compounds which mimic the specific receptor

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interactions of DA better, however, without displaying any intrinsic activity. A new series of compounds were discovered, among which IRL790 was selected for further development.

Preclinical assessment of IRL790 indicated a pharmacokinetic profile enabling oral administration, and no safety concerns, which was corroborated in a Phase 1a trial (IRLAB data on file). IRL790 is currently in clinical development, focusing on LIDs, and PD psychosis. Results from a Phase 1b study in PD were recently published, confirming a favorable safety profile and suggesting improvement of LIDs (Svenningsson et al., 2018).

This paper describes the preclinical pharmacology of IRL790, as investigated in normal and perturbed states including rodent LIDs. The findings are discussed in terms of tentative receptor level mode-of-action, based on *in vitro* assays, along with molecular modelling of GPCR-ligand interactions, focused on the dopamine D3 receptor.

Methods

Animals & drugs

Male Sprague-Dawley rats were purchased from B&K Scanbur (Sollentuna, Sweden; locomotor recordings and tissue analysis in normal, non-pretreated rats), Charles River (Köln, Germany; microdialysis and MK801 interaction study) or Taconic (Ejby, Denmark; AIMs study and d-amphetamine interaction). Rats weighed 160-180 g at the time of arrival, and 220-260 g at the time of the *in vivo* pharmacology studies. Animals were housed five per cage with lights on between 06:00 and 18:00, at 22°C, with free access to food and water. All experiments were carried out in accordance with Swedish animal protection legislation and with the approval of the local Animal Ethics Committee in Gothenburg (EC no 1/2011 (AIMs study), 325/08 (locomotor recordings and tissue analysis), 287/10 (microdialysis)).

Locomotor recordings and *ex vivo* neurochemistry analysis were performed essentially as described in (Waters et al., 2014). IRL790, synthesized in-house as HCl salt, was dissolved in physiological saline (0.9% w/v NaCl) and injected subcutaneously in a volume of 5 ml/kg,

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4 minutes before start of locomotor activity recordings. For interaction studies, rats were pretreated with MK801 (0.7 mg/kg, Sigma-Aldrich) or d-amphetamine (1.5 mg/kg, AKL, Sweden) injected i.p. 90 or 10 minutes, respectively, before start of locomotor recordings.

For the gene map, the following drugs, all purchased from commercial suppliers, were administered: Amantadine HCl (45-405 mg/kg), apomorphine HCl(0.12-1.1 mg/kg), aripiprazole (0.25-2.25 mg/kg), cariprazine HCl (1-3 mg/kg), clozapine (3-30 mg/kg), dihydrexidine HCl(3-10 mg/kg), donepezil (0.37-3.3 mg/kg), memantine HCl (3.3-10 mg/kg), quetiapine fumarate (11-100 mg/kg), risperidone (0.1-1 mg/kg), ropinirole HCl (1.1-10 mg/kg), SCH23390 HCl (0.12-1.1 mg/kg). All drugs were given s.c. in a volume of 5 ml/kg. 60-minute locomotor recordings followed by collection of brain tissue samples were performed as described below, sections “Locomotor recordings” and “Ex vivo neurochemistry”.

Locomotor recordings

Locomotor activity was recorded for 60 min in 55x55 cm sound and light attenuating motility meter boxes, with a manoeuvring space of 41x41 cm (Digiscan activity monitor RZYCCM (16) TAO, Omnitech Electronics, USA.), generating a time series of x, y (horizontal activity) and z (vertical activity) position coordinates sampled at 25 Hz. Distance travelled was calculated based on horizontal coordinates and pooled into 5 min periods. Results are presented as means, with error bars representing SEM. Repeated measures ANOVA with time as the repeated factor, and treatment as a categorical between-group factor, was performed, applying Mauchly's sphericity test and Greenhouse-Geisser and Huyhn-Feldt adjusted significance tests, followed by post-hoc Dunnett's test comparing IRL790 at different doses vs. controls. TIBCO Statistica™ 13.5.0. was used for the statistical analyses.

Ex vivo neurochemistry

Ex vivo neurochemical analysis was performed as described in Waters 2017. Immediately after the behavioural activity recording sessions, animals were decapitated and brains were

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dissected into striatum, cortex and limbic region (containing the nucleus accumbens – both core and shell, most parts of the olfactory tubercle and ventral pallidum). Tissue samples were immediately frozen and stored at -80°C until homogenized with perchloric acid (PCA, 0.1 M), ethylene-diamine-tetraacetic acid (EDTA, 5.37 mM), glutathione (GSH, 0.65 mM) and alpha-methyl-dopamine (0.25 µM) as internal standard. Tissue eluates were analysed with respect to tissue concentrations (ng/g tissue) of the monoamine transmitters NA, DA, 5-HT, and corresponding amine and acid metabolites (NM, 3-MT, DOPAC, HVA, 5-HIAA) by HPLC/EC. The HPLC/EC method is based on two chromatographic separations dedicated for amines or acids. Two chromatographic systems share a common autoinjector with a 10-port valve and two sample loops for simultaneous injection on the two systems. Both systems are equipped with a reverse phase column (Luna C18(2), dp 3 µm, 50*2 mm i.d., Phenomenex) and electrochemical detection is accomplished at two potentials on glassy carbon electrodes (MF-1000, Bioanalytical Systems, Inc.). Neurochemical results are presented as percent of control group means. Error bars = SEM. Statistics: Student's t-test (2 tailed) vs. controls. * denotes $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Microdialysis

In vivo brain microdialysis experiments were performed as described in (Waters et al., 1993; Ponten et al., 2010), with some minor modifications. Microdialysis probes were implanted by stereotaxic surgery 40-48 hours before the experiments, during isoflurane inhalation anaesthesia. Co-ordinates were calculated relative to bregma; dorsal striatum: AP +1.0, ML \pm 2.6, DV -6.2, prefrontal cortex: AP +3.2, ML \pm 1.2, DV -4.0) according to (Paxinos, 1986). After surgery, the rats were housed individually in regular cages, freely moving, throughout the experiment. The perfusion medium for dialysis was a Ringer's solution containing in mmol/l: NaCl; 140, CaCl₂; 1.2, KCl; 3.0, MgCl₂; 1.0, ascorbic acid; 0.04. The rats were perfused for around 60 minutes before baseline sampling began, in order to obtain a balanced fluid exchange. Samples were drawn every 20 minutes. To establish baseline, five fractions of dialysate were collected, and the last three were used to calculate baseline

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levels of each analyte. After establishment of baseline IRL790 was administered s.c. in a volume of 5 ml/kg, with 0.9% NaCl (saline) as vehicle. Vehicle controls, run in a separate experiment, received saline. The monoamine transmitters (NA, DA, 5-HT) as well as their amine (NM, 3-MT) and acid (DOPAC, 5-HIAA, HVA) metabolites were followed for 180 min and quantified by HPLC/EC. After the experiment the rats were uncoupled from the perfusion pump and decapitated. Their brains were rapidly taken out and kept in Accustain one or two days before carefully slicing the brain to verify correct probe position. Only results from rats with correctly positioned dialysis probes were included in the subsequent analysis. Microdialysis data are expressed as mean \pm SEM in percentage of baseline levels. Baseline was defined as the mean level of the three fractions collected immediately before administration of test compound.

In a separate *in vivo* microdialysis experiment, levels of Ach were assessed in extracellular fluid collected from the prefrontal cortex and ventral hippocampus (co-ordinates AP -5.2, ML 4.8, DV -7.5 (Paxinos, 1986), by means of liquid chromatography/tandem mass spectrometry, as described previously (Jerlhag et al., 2012).

Dialysis data were analyzed by a 2-way ANOVA with treatment as independent variable and time as repeated measure. If significant, the 2-way ANOVA was followed by a Fisher's Least Significant Difference Test. Significance between vehicle and IRL790 treatment at specific time-points is indicated in the graphs as follows: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

AIMs

IRL790 was tested in two independent studies, in-house and by an external laboratory, in the 6-OHDA model of AIMs (Lundblad et al., 2005). In this model, the repeated administration of L-DOPA to rats subjected to unilateral 6-OHDA lesions of the nigrostriatal dopaminergic system, elicits adverse involuntary movements, affecting the orofacial region, the limbs and the trunk. The sum of AIMs scored in orofacial region, limbs and trunk is denoted total AIMs, or alternatively, composite AIMs score. Both studies were performed using rats subjected to

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unilateral injections of 6-OHDA (Sigma-Aldrich) into the nigrostriatal fiber bundle, as described previously (Lundblad et al., 2005). In the in-house study, 48 male Sprague-Dawley rats were stereotactically injected with 6-OHDA (10 µg in 2 µl, saline with 0.1% ascorbic acid) into the MFB, in order to achieve complete unilateral dopaminergic denervation. Half of the rats were injected in the right side and the other half were injected in the left side of MFB. Three weeks after lesion animals were subjected to an apomorphine-induced rotation test (0.1 mg/kg i.p.) to verify the success of the lesion procedure. Animals were then given daily injection of L-DOPA at 6.5 mg/kg dose s.c. (plus benserazide 12 mg/kg), for about three weeks, in order to establish full expression of dyskinesias. Twelve rats showing significant dyskinesias were allocated to 2 different groups (n=6/group) balanced to have similar baseline dyskinesia score; one group served as a control group, receiving L-DOPA only, the other group receiving L-DOPA + IRL790, 33 µmol/kg s.c. (*i.e.*, 10 mg/kg) on a separate day of drug test following baseline scoring. Dyskinesias were scored every 20 min (one minute/rat) for the entire time course of L-DOPA (about two hours). Scoring was performed manually by two persons blinded to treatment, and the mean values were used for subsequent statistical calculations and graphs. Limb, axial and orolingual dyskinesias were evaluated individually and summed into composite scores.

In the external study, eighty female Sprague-Dawley rats were stereotactically injected with 6-OHDA (14 µg in 4 µl) into the right MFB, in order to achieve complete unilateral dopaminergic denervation. Three weeks after lesion animals were subjected to the amphetamine-induced rotation test (2.5 mg/kg i.p.) to verify the success of the lesion procedure. Only animals rotating at least 6 turn/min (over 90 min period) were used in the study. Animals were then given daily injections of L-DOPA at 6 mg/kg dose s.c. (plus benserazide 10 mg/kg), for about four weeks, in order to establish full expression of dyskinesias. Thirty-two rats showing significant and stable dyskinesias were allocated to 4 different groups (n=8/group) balanced to have similar baseline dyskinesia scores; one group served as control (L-DOPA only), the other groups received L-DOPA + IRL790 at three

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different doses. Dyskinesias were scored every 20 min for the entire time course of L-DOPA (about two hours). Limb, axial and orolingual dyskinesias were evaluated individually and the composite score was then calculated. IRL790 was initially tested at 1, 3 and 10 mg/kg s.c., given 20 min before L-DOPA (n=8 rats/group). Subsequently, the effects of 20 mg/kg s.c were tested in a separate experimental session (n=7 rats/group, controls received L-DOPA only).

Graphs display means of composite dyskinesia scores, with errors bars representing SEM. Graphs with medians and quartiles are provided as supplementary data. In the external study, a repeated measures ANOVA was performed on the composite scores, applying Mauchly's sphericity test and Greenhouse-Geisser and Huyhn-Feldt adjusted significance tests, followed by post-hoc Dunnett's test comparing IRL790 at different doses vs. controls. The results from the test of 20 mg/kg + L-DOPA vs L-DOPA treated controls were analysed separately. The results were confirmed with non-parametric tests; Kruskal-Wallis test for the initial dose response study, and Mann-Whitney U-test comparing 20 mg/kg vs L-DOPA controls, based on total composite scores. An integrated dose response analysis using total composite scores collected from both experimental sessions was undertaken, fitting a sigmoid curve to the data, using the TIBCO Spotfire software. In the in-house study, statistical comparison between control and IRL790 treatment were performed using ANOVA with time as repeated measure, confirmed with Mann-Whitney U-test, based on total composite scores.

Analysis of mRNA

For *ex vivo* mRNA analysis, brains were removed immediately following the completion of locomotor recordings and were dissected into four regions: Limbic system (containing nucleus accumbens, most parts of the olfactory tubercle, ventral pallidum and amygdala), striatum, frontal cortex and hippocampus. Tissue samples were stored at -80°C until further processing. Total RNA was prepared using the guanidine isothiocyanate method (Chomczynski and Sacchi, 1987). RNA pellets were dissolved in RNase-free water and

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stored at -80°C. The RNA concentration was determined spectrophotometrically using a NanoDrop ND-1000 (Saveen Werner, Limhamn, Sweden) and the quality and integrity of random samples were checked using an Experion automated electrophoresis system (Bio-Rad, Sundbyberg, Sweden). Reverse transcription was performing using a SuperScript III kit (Invitrogen, Groningen, Netherlands) as follows: 1 µg of total RNA was mixed with 5 µl 2 x RT Reaction Mix and 1 µl of RT Enzyme Mix. The reaction volume was adjusted to 10 µl with RNase-free water. The mixture was incubated at 25°C for 10 min, 50°C for 30 min then 85°C for 5 min. Escherichia coli RNase H (1U) was added and the reaction mixture was incubated at 37°C for 20 min then 85°C for 5 min. The final cDNA was diluted 40 times in 10 mM Tris-HCl, 0.1 mM EDTA pH 7.8 and stored at -20°C.

Single-plex real-time PCR

For all genes, 0.7 µl of cDNA solution was incorporated in a reaction mixture containing 1 x PCR buffer, 0.2 mM dNTP, 3.7 mM magnesium chloride, 0.15 mM SYBR green, 0.4 µM of each primer and 1 U of Taq polymerase (Invitrogen, Stockholm, Sweden) in a final volume of 25 µl. Real-time PCR was performed using a CFX96 Real-Time PCR Detector (Bio-Rad, Sundbyberg, Sweden) with the following cycling conditions: initial denaturation at 95°C for 1 min followed by 40 cycles of 95°C for 10 s, 56°C for 10 s and 72°C for 10 s. Primer sequences were as follows HPRT (accession number NM 012583), sense 5'-GGC CAG ACT TGT TGG ATT TG-3', antisense 5'-CCG CTG TCT TTT AGG CTT TG-3', cyclophilin A (accession number M19533), sense 5'-GTC TCT TTT CGC CGC TTG CT-3', antisense 5'-TCT GCT GTC TTT GGA ACT TTG TCT G-3', Arc (accession number U19866), sense 5'-GTC CCA GAT CCA GAA CCA CA-3', antisense 5'-CCT CCT CAG CGT CCA CAT AC-3', cfos (proto-oncogene accession number NM 022197), sense 5'-CAG AGC ATC GGC AGA AGG-3', antisense 5'-AGT TGA TCT GTC TCC GCT TGG-3. The sample DNA concentration was estimated using a standard curve constructed for each gene using serial dilutions of purified PCR products. Correct PCR products were identified by agarose gel electrophoresis, purified using the PCR Purification Kit (Qiagen, Sollentuna, Sweden),

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sequenced at MWG-Biotech AG (Ebersberg, Germany) and analysed routinely by melting-curve analysis to confirm the specificity of the reaction. Yields of Arc and cfos genes were normalized using the geometric mean of the yields of HPRT and cyclophilin A.

TaqMan single and duplex PCR

The real-time PCR reaction consisted of 10 µl Sso Advanced Universal Probes Supermix, 1 µl primer/probe, 1 µl reference gene or 1 µl MQ water and 8 µl of cDNA (diluted 40 times from RT-PCR). Real-time PCR reactions were performed in a CFX96 Real-Time PCR Detector (Bio-Rad, Sundbyberg, Sweden) with the following cycling conditions: initial denaturation at 95°C for 2 min followed by 40 cycles of 95°C for 5 s and 60°C for 30 s. All genes of interest were labelled with the fluorophore FAM on the 5' end and reference genes were labelled with HEX. TaqMan primers and probes were synthesized by Bio-rad (Coralville, Iowa, USA) and used according to the manufacturing protocol. The gene *homer1a* (in house designed, GLGF-domain protein accession number U92079.1) sense 5'-TGGCATAATACCTTACCTTGAGT-3', antisense 5'-GCACTCTACAG TATATACGAGCC-3', Probe Sequence: TTGGTTTCTAAAACTCTACTGCT (Bio-Rad (Coralville Iowa; USA) was analyzed in duplex with the reference gene *Ppia* (cyclophilin A peptidyl-propyl cis-trans isomerase qRnoCIP0050815). *Egr1* (Early growth response protein 1, BioRad Assay ID qRnoCEP0022872, chromosome location 18:27371314-27371422) was analyzed in duplex with the reference gene *HPRT* (qRnoCEP0050840). Neuronal *Nptx2* (pentraxin 2 qRnoCEP0030302) and *Npas4* (neuronal PAS domain protein4 qRnoCEP0029461) were both analyzed in singleplex. *Ppia* and *HPRT* were used for normalization.

Results are presented as means ± SEM, expressed as percentages of control group means. Statistical significance was assessed using Student's t-test (2-tailed) vs. Controls: * p<0.05, ** p<0.01, *** p<0.001.

For the gene mapping on multiple compounds, multivariate analysis was performed as described in (Waters et al., 2017), applying PLS regression analysis using a discriminant

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variable block denoting doses of test compounds as dependent (Y) variable block (n Y variables), and mRNA levels in each brain region analysed, as independent variable block (4x6=24 X variables). The independent variables were subject to zero mean and unit variance scaling, and block scaled by IEG. Statistical significance was determined by cross-validation. Multivariate analyses were carried out using SIMCA 16.0 (Umetrics, Inc.). Results are visualized as w*c loading plots showing clusters and effect patterns among the test compounds analysed.

In vitro pharmacology

All *in vitro* pharmacology studies were carried out using standard assays by the laboratories of CEREP, France, (see, <https://www.eurofindiscoveryservices.com/cms/cms-content/services/in-vitro-assays/>). A broad binding screen (ExpresSProfile Panel, 55 targets, list provided as Supplementary data) covering receptors, enzymes and transporters related to all major CNS neurotransmitters, was performed at 10 μ M IRL790, run in triplicates at each binding target, followed by determination of K_i /IC₅₀ at selected targets displaying at least \approx 20% inhibition of control specific binding. The specific ligand binding to the receptors is defined as the difference between the total binding and the nonspecific binding determined in the presence of an excess of unlabelled ligand. Inhibition of control specific binding was calculated as $(100 - ((\text{measured specific binding} / \text{control specific binding}) \times 100))$ obtained in the presence of the test compound (IRL790). For IC₅₀/K_i determination, IRL790 was tested at 8 concentrations, in duplicates. IC₅₀ values (concentration causing a half-maximal inhibition of control specific binding) were determined by non-linear regression analysis of the competition curves generated with mean replicate values using Hill equation curve fitting. The inhibition constants (K_i) were calculated using the Cheng-Prusoff equation ($K_i = IC_{50} / (1 + (L / K_D))$, where L = concentration of the radioligand in the assay, and K_D = affinity of the radioligand for the receptor). Source of cells were either Chinese hamster ovary (CHO) or human embryonic kidney293 (HEK-293). Details of specific binding assays are given in Table 1.

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For selected receptor targets, additional *in vitro* studies were performed, assessing intrinsic activity, and antagonist properties. Assay details are provided in table 2.

EC₅₀ values (concentration producing a half-maximal response) and IC₅₀ values (concentration causing a half-maximal inhibition of the control agonist response) were determined by non-linear regression analysis of the concentration-response curves generated with mean replicate values using Hill equation curve fitting: $Y = D + [A - D] / [1 + (C / C_{50})^{nH}]$ where Y = response, A = left asymptote of the curve, D = right asymptote of the curve, C = compound concentration, and C₅₀ = EC₅₀ or IC₅₀, and nH = slope factor. This analysis was performed using software developed at Cerep (Hill software) and validated by comparison with data generated by the commercial software SigmaPlot® 4.0 for Windows® (© 1997 by SPSS Inc.). For the antagonists, the apparent dissociation constants (K_B) were calculated using the modified Cheng Prusoff equation $K_B = IC_{50} [1 + (A / EC_{50A})]$ where A = concentration of reference agonist in the assay, and EC_{50A} = EC₅₀ value of the reference agonist.

Computational methods

The development of a structure model of the D2 receptor and the docking of IRL790 into the ligand binding site of dopamine D3 and D2 receptor structures was performed using the MOE software (Molecular Operating Environment, version 2019.01, Chemical Computing Group Inc., Montreal, QC, Canada). The X-ray crystal structure of the human dopamine D3 receptor (PDB entry 3PBL) was used for docking of IRL790 and as template for the development of a homology model of the human dopamine D2 receptor structure (Chien et al., 2010). The recently published dopamine D2 receptor structure complex with the antagonist risperidone PDB entry 6CM4 (Wang et al., 2018a) was evaluated and disregarded as a structure model due to the atypical conformation of the receptor induced by risperidone. The c-terminal part of the second extracellular loop (ECL2) that constitutes the upper part (or the lid) of the orthosteric binding site adopts a unique open conformation in

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the D2R structure, not present in the other 39 solved X-ray structures of monoaminergic GPCRs with a resolution ≤ 3.0 Å.

Results

In vivo pharmacology

The pharmacological properties of IRL790 were investigated in a series of *in vivo* studies in rats, including models of psychosis and AIMS, based on disrupted dopaminergic or glutamatergic neurotransmission. Neurochemical biomarkers collected include monoaminergic indices assessed in brain tissue *ex vivo*, and by *in vivo* brain microdialysis, as well as IEGs.

Effects on behavior

In normal, non-pretreated rats, there was no significant effect on locomotor activity at 3.7-100 $\mu\text{mol/kg}$ (significant effect of time, $F(11,165)=61.1$, adjusted $p<0.00001$, but not of treatment or time*treatment; Figure 1). IRL790 was further investigated in two pharmacological rat models of psychosis, *i.e.* in rats displaying aberrant, hyperactive motor behaviour due to pre-treatment with the catecholamine releaser d-amphetamine (hyperdopaminergic state) or the glutamatergic NMDA receptor antagonist (+)-MK-801 (hypoglutamatergic state). IRL790 dose dependently inhibited the behavioural activation following pretreatment with d-amphetamine or MK-801 (Figure 2 and 3). In d-amphetamine pretreated rats, there was a significant effect of treatment ($F(4,15)=18.918$, $p=0.00001$), time ($F(11,165)=36.23$, $p<0.00001$), and time*treatment ($F(44,165)=2.35$, $p=0.00005$, adjusted $p<0.05$). Post-hoc analysis indicated significant inhibition of locomotion at 33 $\mu\text{mol/kg}$ s.c ($p<0.001$), and 100 $\mu\text{mol/kg}$ s.c ($p<0.001$). Similar effects were observed in rats pretreated with MK801; with a significant effect of treatment ($F(4,15)=10.1$, $p=0.00035$), time ($F(11,165)=53.9$, $p<0.000001$), and time*treatment ($F(44,165)=3.09$, $p<0.000001$, adjusted $p<0.05$). Post-hoc analysis indicated significant reductions at 33 $\mu\text{mol/kg}$ s.c ($p<0.05$), and 100 $\mu\text{mol/kg}$ s.c ($p<0.01$). These pharmacological effects were also present upon oral administration of similar doses of IRL790 (not shown).

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The potential anti-dyskinetic effects of IRL790 were investigated in rats subjected to unilateral 6-OHDA lesions of the nigrostriatal dopaminergic system, combined with repeated treatment with L-DOPA, which elicits adverse involuntary movements affecting the orofacial region, the limbs and the trunk. Rat AIMs present many functional and phenomenological analogies to L-DOPA induced dyskinesia in patients with PD (Cenci and Lundblad 2005). IRL790 was tested in the 6-OHDA model in two independent studies, one run in-house and one run by an external laboratory (University of Cagliari).

Tested at IRLs laboratories, IRL790 at 33 $\mu\text{mol/kg}$ s.c. resulted in a near complete suppression of total AIMs, which persisted during the full 2-hour experiment period (Figure 4). Repeated measures ANOVA indicated a significant effect of time ($F(6,60)=28,729$, $p<0.000001$), treatment ($F(1,10)=125,21$, $p<0.000001$), and treatment * time ($F(6,60)=17,416$, $p<0.000001$). Non-parametric analysis confirmed statistical significance ($p=0.005$ vs. Controls based on total composite scores). The total composite score was 8.7 ± 3.3 points in the IRL790 + L-DOPA group, and 56.8 ± 2.8 in controls receiving L-DOPA only. (Baseline total composite scores were 47.9 ± 5.1 and 48.1 ± 5.5 , in the IRL790 and control groups, respectively). Limb, orolingual and axial AIMs were all equally reduced by IRL790 (not shown).

In the external study, IRL790 was initially tested at 1, 3 and 10 mg/kg s.c., corresponding to 3.2, 9.6 and 32 $\mu\text{mol/kg}$ s.c, administered 20 min before L-DOPA ($n=8$ rats/group). As IRL790 did not appear to reduce the L-DOPA-induced motor activation in the test cages, a fourth dose of 20 mg/kg (64 $\mu\text{mol/kg}$) was tested in a separate session ($n=7$ rats/group). A dose-dependent anti-dyskinetic effect was observed with a reduction by approximately 40% in the total composite AIMs score as compared to the control group at 10 mg/kg, and a 45% reduction at 20 mg/kg (Figure 5). The repeated measures models indicated a significant effect of time ($F(6,168)=93.3$, $p<0.000001$) and treatment ($F(3,28)=6.2$, $p=0.002$) in the dose response experiment as well as in the experiment testing only 20 mg/kg vs L-DOPA (Time $F(6,72)=43$, $p<0.000001$, Treatment $F(1,12)=48.1$, $p=0.00002$, Time*Treatment

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$F(6,72)=11.8$, $p<0.000001$, adjusted $p=0.000006$. Post-hoc testing indicated significant effects at 10 mg/kg, $p=0.0021$, and 20 mg/kg, $p=0.000024$. Statistical significance was confirmed with non-parametric testing, showing a significant effect at 10 mg/kg, $p=0.017$ vs controls, and at 20 mg/kg, $p=0.002$ vs controls. At the highest dose, L-DOPA- induced rotation was slightly increased in the L-DOPA + IRL790 treated group suggesting that the anti-dyskinetic effect of IRL790 did not reduce the beneficial L-DOPA induced motor activation (Figure 6). Taken together, combining the two experimental sessions covering the dose range 1-20 mg/kg (3.2-64 $\mu\text{mol/kg}$) s.c, a dose dependent reduction of LIDs was observed, with an ED50 of ~ 4 mg/kg (13 $\mu\text{mol/kg}$; Figure 7), with a reduction by 40 to 50% in the total composite AIMS score as compared to the control group at 10 and 20 mg/kg.

Effects on neurochemistry and IEGs

Ex vivo neurochemical analysis of brain tissue revealed dose dependent effects on monoaminergic indices, in particular DOPAC and HVA, reaching approximately 250-300% relative to control group means at the highest dose (100 $\mu\text{mol/kg}$) in all three brain structures sampled, suggesting increased dopamine metabolism across brain regions (Figure 8). There were also decreases in cortical NA and DA, at the top dose (reduced to 80% and 85% of control mean, respectively, $p<0.05$), and in striatal DA at 33 and 100 $\mu\text{mol/kg}$ (83%, $p<0.05$ and 78%, $p<0.001$, respectively, relative to controls).

Neurochemical effects were also assessed by *in vivo* brain microdialysis, capturing monoamine analytes and Ach in extracellular fluid in conscious, freely moving rats. Dialysates were collected from the striatum, and the prefrontal cortex, for 3 hours following the administration of IRL790, at 16.7 or 50 $\mu\text{mol/kg}$ s.c., and assessed with respect to monoaminergic neurochemical indices. In a separate microdialysis study, Ach levels were monitored in prefrontal cortex, ventral hippocampus and striatum, following IRL790 50 $\mu\text{mol/kg}$ s.c or saline. IRL790 elicited dose dependent increases in extracellular levels of NA and DA (Figure 9), as well as the DA metabolites DOPAC and HVA (not shown). At 50 $\mu\text{mol/kg}$, prefrontal cortex DA increased to 200% of baseline, and striatal DA reached 270%

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of baseline. NA increased to 130% of baseline in the prefrontal cortex, and 270% of baseline in the striatum. Repeated measures ANOVA indicated a significant effect of time ($F(9,117)=9.46$, $p<0.000001$), and time*treatment ($F(18,117)=2.57$, $p=0.001$) for DA in the prefrontal cortex, a significant effect of time ($F(9,126)=15.44$, $p<0.0000001$), treatment ($F(2,14)=17.96$, $p=0.0001$), and time*treatment for DA in the striatum, ($F(18,126)=5.67$, $p<0.0000001$), and a significant effect of time ($F(9,63)=3.08$, $p<0.004$), and time*treatment for NA in the striatum ($F(18,63)=2.49$, $p=0.004$). There was no significant effect on 5-HT in either region. Levels of Ach were increased across brain regions, reaching 329 % of baseline in the pfc and 225 % of baseline in the ventral hippocampus (Figure 10). Repeated measures ANOVA indicated a significant effect of time ($F(9,90)=12.37$, $p<0.000001$), treatment ($F(1,10)=33.14$, $p=0.0002$), and time*treatment ($F(9,90)=4.85$, $p=0.00003$) in the pfc as well as in the ventral hippocampus (time ($F(9,36)=4.45$, $p=0.0006$), treatment ($F(1,4)=18.5$, $p=0.013$), time*treatment ($F(9,36)=2.26$, $p=0.040$).

Effects on IEG mRNA are shown in figure 11. IRL790 dose dependently and potently increased *Arc* in the striatum, reaching 241% ($p<0.01$), 329% ($p<0.001$), and 384% ($p<0.001$) vs. vehicle controls at 11, 33, 100 $\mu\text{mol/kg}$, respectively. *Arc* was also significantly increased at 11-100 $\mu\text{mol/kg}$ in the frontal cortex and in the limbic region, reaching 153-162% of control in the frontal cortex and 166-186% of control in the limbic region (Figure 10). *Cfos*, *homer* and *egr* were increased in the striatal and limbic regions. *NPAS4* was increased in the striatum. As with *Arc*, the effects on *cfos*, *homer*, *egr*, and *NPAS4* were generally potent, reaching statistical significance even at the lowest dose tested (11 $\mu\text{mol/kg}$), with particularly large magnitudes for striatal *cfos*, *homer* and *NPAS4* (300-800% of control means at the top dose). There were also minor effects on *Nptx2*, however of small magnitude and with no consistent dose response pattern: small increases were observed in the striatum at 33 and 100 $\mu\text{mol/kg}$ (30-50% increase vs. controls), and a slight decrease in the frontal cortex (around 20% decrease vs. controls at 11 and 33 $\mu\text{mol/kg}$).

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For the gene map, IEG expression data on 13 compounds were analysed using PLS regression, as described previously (Waters et al., 2017). A five-component model was obtained, describing 72% of the variance in the X-block (R^2X), and 26% of the variance in the Y-block (R^2Y), $Q^2_{cum} = 0.184$. W^*c loadings for the two first components are shown in Figure 12. This type of plot shows clusters among the compounds studied, along with underlying effect patterns in the variables measured. Compounds located close to each other in the plot tend to share gene expression patterns, while diametrically opposing positions indicated opposing effects. For the variable w^* loadings, clustering indicates covariance, and diametrically opposing positions reflects negative correlations. Positions in orthogonal directions indicates independent effects. In this model, the first component (X-axis) essentially represents overall increase (left) or decrease (right) across IEGs, while component 2 (Y-axis) captures regional differences (increase in striatal region downwards, cortical regions upwards). There is a clustering of antipsychotic compounds, in the lower right quadrant, reflecting a shared feature of general decreases in IEGs, along with a tendency to increase striatal Arc and cfos (downward direction in plot). Antiparkinsonian agents (ropinirole, amantadine) display weak effects, decreasing striatal IEGs, and, for ropinirole in particular, increases in cortical IEGS such as NPAS4 and cfos. Apomorphine, donepezil and memantine all display more pronounced increases in cortical/hippocampal IEGs, but less effects on striatal IEGs. IRL790 displays a distinct pattern, with increases both in striatal, limbic and cortical IEGs, in particular Arc, resulting in an intermediate position between antipsychotics and the DA D1 agonist, dihydrexidine.

In vitro pharmacology

In vitro binding characteristics of IRL790 were assessed in a broad radioligand binding screen, followed by determination of IC₅₀/K_i values at selected targets, at which at least 20% inhibition of control specific binding was observed (Table 1).

IRL790 displayed moderate affinity across a range of receptors, enzymes and transporters (Table 1). At the primary target, the dopamine D3 receptor, IRL790 had a K_i of 90 nM. Sub-

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micromolar affinities were also measured at DA D2 and Sig-1R (σ_1) receptors, and micromolar at 5-HT1a, 5-HT2a, and 5-HT7 receptors. IRL790 was further investigated *in vitro* in assays designed to detect intrinsic activity in cellular functional assays at human recombinant D2S, D3 and serotonergic receptors (see Table 2). IRL790 did not show any agonist like properties at D2, D3, 5-HT2 or 5-HT7 receptors. The compound displays weak partial agonist properties at 5-HT1A receptors, albeit with an EC50 > 100 μ M.

IRL790 was also tested for antagonist properties in *in vitro* cellular assays with human recombinant receptors. Antagonist properties at DA D2S, 5-HT1A and 5-HT2A receptors were observed, see Table 3.

Computational docking studies

Figure 13 shows the best scoring binding pose for IRL790 in the crystal structure of the dopamine D3 receptor (Chien et al., 2010). IRL790 binds in a horizontal orientation with reference to the vertically oriented transmembrane helices. The methyl-sulfone moiety of IRL790 points towards the serine residues Ser192 and Ser193 ($S^{5.42}$ and $S^{5.43}$ using Ballesteros–Weinstein numbering; (Ballesteros and Weinstein, 1995) in the transmembrane helix 5 (TM5) and the basic amine interacts with the aspartic acid residue Asp110 ($D^{3.32}$) in TM3. These conserved residues among monoaminergic G-protein coupled receptors are known to be key interaction residues for receptor agonists (Malo et al., 2012). Thus, the modelling results indicate that IRL790 preferably binds in a typical agonist-like binding mode. The best scoring binding pose for IRL790 in the structure model of the dopamine D2 receptor is in essence similar to the best D3 pose, but the internal conformational energy of IRL790 is higher and the docking score slightly worse for the D2 pose. The only sequence position in the binding site region which differ between the D2 and D3 receptors is in the second extracellular loop (ECL2) at the C+1(Conner et al., 2007) position, *i.e.*, the position after the conserved cysteine residue making a disulfide bridge to TM3, which is close to the binding pocket entrance (See Figure 14). The D3 receptor has a Serine pointing away from the ligand (IRL790) in the crystal structure and the D2 receptor has a bulkier isoleucine

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amino acid which is pointing towards the ligand and causing the entrance to be narrower in the D2 structure model.

Discussion

IRL790 was developed with the aim to create an antidyskinetic and antipsychotic compound, exerting its pharmacological effects through interactions with DA D3/D2 type receptors. The underlying design strategy was to use full or partial dopamine receptor agonists and modify their pharmacological properties away from agonism towards antagonism without following the conventional route of increasing size and lipophilicity. Given that agonists of DA D3/D2 receptors tend to be smaller, more hydrophilic molecules whereas antagonists are usually larger and more lipophilic (Malo et al., 2010; Li et al., 2016), we speculated that this approach could yield DA D3/D2 antagonists which mimic the specific interactions of DA (i.e., active state of the receptor) better than large, lipophilic antagonists, which binds to and stabilize the inactive state of the receptor (Goddard and Abrol, 2007), with slow receptor dissociation (Tresadern et al., 2011). We hypothesized that such a dopamine antagonist with physicochemical properties similar to agonists would exert antidyskinetic and antipsychotic effects in states of dysregulated dopaminergic signaling, while having little negative impact on physiological dopamine transmission and, hence, minimal liability for motor side effects. This prediction was to a large extent manifested in IRL790s *in vivo* pharmacological profile. The starting point for discovery and development of IRL790 was a series of 2-(aminomethyl)-chromans (Mewshaw et al., 1997) as partial DA D2 agonists with varying intrinsic activity. By careful modification of key elements important for intrinsic activity, a series of compounds with DA D3/D2 receptor antagonist properties were discovered, among which IRL790, a small, hydrophilic compound (clogP=1.59, MW=275.3) was selected for further development. The *in vivo* test battery was performed in male rats, except for the external AIMs study, where female rats were used.

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IRL790 displays a neurochemical signature with effects indicating increased dopamine metabolism across brain regions. In line with the effects on *ex vivo* monoaminergic neurochemistry, *in vivo* microdialysis showed increases in extracellular levels of NA and DA in the frontal cortex and the striatum. Similar profiles have been observed with atypical antipsychotics, attributable to dopamine receptor antagonism, known to produce increases in DOPAC and HVA (Carlsson and Lindqvist, 1963; Magnusson et al., 1986), in combination with interactions at 5HT1a, 5HT2a, 5HT7, or alpha2 receptors, proposed to result in enhanced release of dopamine (Alex and Pehek, 2007; Wesolowska and Kowalska, 2008; Marcus et al., 2010). IRL790 also increased extracellular levels of ACh in the pfc and hippocampus, an effect observed with DA D3 antagonists, and some atypical, but not typical antipsychotics (Lacroix et al., 2006). The gene expression profile also shares some features with antipsychotics. *Arc* is an immediate early gene triggered by, e.g., synaptic NMDA receptor activation (Bramham et al., 2010). It is involved in consolidation of memory and related processes, such as long-term potentiation (LTP) and is a key regulator of synaptic plasticity. *Cfos*, encoding a transcription factor, is a more non-specific marker of neuronal activation (de Bartolomeis et al., 2017). The dose dependent increase of striatal *Arc*, and *cfos*, is a common feature of dopamine D2 antagonists (Robbins et al., 2008; Fumagalli et al., 2009; Waters et al., 2014; de Bartolomeis et al., 2017), proposedly linked to antagonism at DA D2 heteroceptors at glutamatergic cortico-striatal terminals, leading to enhanced synaptic NMDA mediated signaling onto striatal neurons (Waters et al., 2014). With IRL790, we also see dose dependent increases of *Nptx*, *NPAS*, *egr*, and *homer* in the striatum, likely reflecting downstream effects of the modulation of synaptic activity in striatal neurons. The increase in frontal cortex *Arc*, on the other hand, is not shared by dopamine antagonists, which generally decrease this biomarker upon acute administration (Waters et al., 2014; de Bartolomeis et al., 2017). Enhanced cortical ACh may contribute to this cortical *Arc* increase (Teber et al., 2004). The gene map provides a comparative overview of IEG expression patterns for a set of compounds relevant for the PD population. The IEG profile of IRL790 is reflected in a position in the map representing both features shared with antipsychotics; as

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discussed above, and features unique to IRL790 e.g., increases in frontal and limbic IEGs (*Arc*, *cfos*, *NPAS4*). This profile is distinct from the other classes represented in the model, including cariprazine, a novel antipsychotic described as a DA D3- vs. D2-receptor-preferring partial agonist (Garnock-Jones, 2017) that is more akin to other antipsychotics in its IEG expression profile. Moreover, IRL790 displays antagonist properties at the D3 receptor, and thus differs from cariprazine which is a high affinity partial agonist at these sites. This notwithstanding, IRL790 paradoxically displays an, agonist-like receptor binding mode (see docking studies below). Taken together, the binding properties imply a different interplay with the endogenous ligand at D3 receptors, as compared to cariprazine, in turn likely influencing down-stream effects measured *in vivo*, including IEGs. IRL790 is also distinct from compounds used in dementia, which generally show their most prominent IEG increases in frontal cortex and hippocampus, with less impact on striatal IEGs.

The effects of IRL790 on locomotor activity in rodents were studied in several models. There was no effect on spontaneous locomotor activity over a dose range eliciting significant effects on neurochemical and gene biomarkers. In contrast, IRL790 reduced locomotor hyperactivity elicited by pretreatment with either d-amphetamine or MK-801, demonstrating the ability to normalize the behavioural phenotype in hyperdopaminergic as well as hypoglutamatergic states.

Balancing effects on aberrant motor phenotypes were also evident in the investigations made using the rodent 6-OHDA AIMs model. This model is considered to have predictive validity for evaluation of anti-dyskinetic efficacy of new therapies (Inderberg et al., 2012). IRL790 dose dependently reduced AIMs, and this alleviation of involuntary movements was not achieved at the cost of any impairment of the motor effects of L-DOPA as such, in this study captured as rotational response to L-DOPA. It should be noted that such rotations are a complex phenomenon, not solely representing anti-parkinsonian effects of L-DOPA; however, the lack of reduction of rotations appears consistent with the lack of general

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inhibition of locomotor activity by IRL790. At the highest dose of IRL790, rotational behaviour even appeared somewhat enhanced.

In vitro, sub-micromolar affinities were observed at DA D3, D2 and σ 1- receptors, and micromolar affinities at 5-HT1a, 5-HT2a, and 5-HT7 receptors. Functional *in vitro* studies showed no intrinsic activity at the receptors studied, except at 5HT1A receptors where a partial agonist effect at high concentrations, EC50 \approx 100 μ M, was detected. Additional functional studies confirmed antagonist actions of IRL790 at DA D3, D2, 5HT1a and 5HT2a receptors. The highest affinity was found at DA D3, followed by D2 (6-8 fold less) receptors.

Dopamine D3 receptors are clearly implicated in the pathogenesis of LIDs, where they are hypothesized to mediate aberrant dopamine D1 receptor signaling, through the formation of D1-D3 receptor dimers (Solis and Moratalla, 2018). Hence, D3 receptor antagonism has been suggested as an attractive target for pharmacological alleviation of LIDs. Peak plasma concentrations of IRL790 reported from a clinical PD-LIDs study, around 220 nM, are consistent with pharmacologically relevant interactions at DA D3 receptors in the suggested therapeutic dose range (Svenningsson et al., 2018).

D3/D2 receptors are also implicated in psychosis treatment, with most therapeutic agents being D2-preferring receptor antagonists or partial agonists. Interestingly, the D3/D2 preference ratio displayed by IRL790 is similar to that reported for the recently launched partial DA receptor agonist cariprazine, which has shown broad antipsychotic effectiveness (Garnock-Jones, 2017; Nemeth et al., 2017; Fleischhacker et al., 2019). IRL790 is less potent at D3 receptors than cariprazine, but is nonetheless within the same nanomolar affinity range as the endogenous transmitter DA itself for this site (Kiss et al., 2010; Stahl, 2017). IRL790 would therefore be expected to effectively compete with DA for the D3 receptors *in vivo* at exposures attained under the conditions reported in this paper.

Computational docking studies of IRL790 into the ligand binding site of dopamine D3 and D2 receptor structures confirm the design idea of this new type of dopaminergic antagonist with an agonist like binding mode (Figure 13). The methyl sulfone moiety of IRL790 does not

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interact with the serine residues in TM5 in the same way as the typical catechol moiety in agonists does. This may explain the lack of intrinsic activity observed for IRL790. Shape differences in the entrance region of the ligand may explain the differences in docking scores and ligand conformational energies observed when docking IRL790 into dopamine D3 vs. D2 receptor models. The combination of an agonist like binding mode of IRL790 and the subtle difference in the shape of the binding sites of the D3 and D2 receptors is consistent with the moderate *in vitro* D3 binding affinity and the D3 vs. D2 preference observed.

In summary, IRL790 displays an *in vivo* pharmacological effect pattern with reduction of AIMS in the rodent 6-OH-DA lesion model, and anti-psychotic like effects in validated pharmacological models, without negatively impacting normal motor performance. The *in vivo* profile, including neurochemical and IEG responses suggesting modulation of DA neurotransmission, is consistent with the *in vitro* pharmacology suggesting DA D3 receptors as a primary site of action. Computational docking at DA D3 receptors supports preferential affinity at DA D3 receptors, with an agonist like binding mode. This said, the precise extent to which the DA D3, D2 and other target sites contribute to the overall profile of IRL790 remains to be further established. The *in silico*, *in vitro* and *in vivo* properties of IRL790 reflect the design principle applied in the drug discovery process, where we sought to obtain antagonist compounds with an agonist like structural motif, in order to softly modulate endogenous neurotransmission, in such a way that normal dopaminergic functions are left intact, while motor and psychiatric disturbances related to aberrations in dopaminergic transmission are alleviated. This strategy, we believe, is a key factor in the favorable clinical efficacy and tolerability profile of IRL790, even in the very sensitive population of patients with advanced Parkinson disease (Svenningsson et al., 2018).

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Authorship contributions

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Conducted experiments: Edling, Gunnergren, Svanberg, Ljung, Carta, Fagerberg.

Performed data analysis: Edling, Fagerberg, Gunnergren, Kullingsjö, Ljung, Svanberg, Svensson, Waters, S.

Wrote or contributed to the writing of the manuscript: Carta, Edling, Fagerberg, Gunnergren, Hjorth, Kullingsjö, Ljung, Sonesson, Svanberg, Svensson, Tedroff, Waters, N, Waters, S.

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Figure legends

Figure 1. Effects of IRL790 on spontaneous locomotor activity. Shown are means±SEM of total distance travelled, over 5-minute intervals throughout the 60-minute recording session, expressed in arbitrary length units. IRL790 (3.7, 11, 33 or 100 µmol/kg, s.c.) was administered s.c. 4' before the start of recording. Controls received saline. N=4/group. There was a significant effect of time ($p<0.00001$), but no significant effect of treatment, or time*treatment (repeated measures ANOVA).

Figure 2.

Effects of IRL790 on d-amphetamine induced hyperactivity. Shown are means±SEM of total distance travelled, over 5-minute intervals throughout the 60-minute recording session, expressed in arbitrary length units. D-amphetamine (1.5 mg/kg i.p.) was administered 10' before, and IRL790 (3.7, 11, 33 or 100 µmol/kg, s.c.) was administered 4' before the start of recording. Controls received saline. N=4/group. IRL790 significantly reduced d-amphetamine induced hyperactivity at 33 and 100 µmol/kg ($p<0.001$, repeated measures ANOVA, post-hoc Dunnett's test).

Figure 3.

Effects of IRL790 on MK801-induced hyperactivity. Shown are means±SEM of total distance travelled, over 5-minute intervals throughout the 60-minute recording session, expressed in arbitrary length units. MK-801 (0.7 mg/kg i.p.) was administered 90' before, and IRL790 (3.7, 11, 33 and 100 µmol/kg, s.c.) 4' before the start of recording. Controls received saline. N=4/group. IRL790 significantly reduced MK-801 induced hyperactivity at 33 ($p<0.05$) and 100 ($p<0.01$) µmol/kg (repeated measures ANOVA, post-hoc Dunnett's test).

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Figure 4.

Effects of IRL790 in the AIMS model, in-house study. Rats were subjected to unilateral 6-OHDA lesion followed by three weeks treatment with L-DOPA, 6.5 mg/kg dose s.c. (plus benserazide 12 mg/kg), in order to establish full expression of dyskinesias. On the test day IRL790 (33 μ mol/kg s.c.) was administered together with L-DOPA (6.5 mg/kg s.c.) (n=6). Controls received L-DOPA only (n=6). Groups were balanced to have similar AIMS scores at baseline. Shown are means \pm SEM of composite AIMS scores, measured at 20-minute intervals. IRL790 resulted in a near complete suppression of dyskinesia which was statistically significant (Effect of treatment, $p < 0.000001$, repeated measures ANOVA; $p < 0.01$, Mann-Whitney U-test, based on total composite scores)

Figure 5.

Effects of IRL790 in AIMS model, external study.

Rats were subjected to unilateral 6-OHDA lesion followed by four weeks treatment with L-DOPA (6.5 mg/kg dose s.c., plus benserazide 12 mg/kg s.c.), in order to establish full expression of dyskinesias. On the test day IRL790 (1, 3 or 10 mg/kg mg/kg, s.c.) was administered 20' before L-DOPA (6.5 mg/kg s.c.) (n=8). Controls received L-DOPA only (n=8). In a separate experimental session, L-DOPA+IRL790, 20 mg/kg, was tested (n=7/group). All groups were balanced to have similar AIMS scores at baseline. Shown are means \pm SEM of composite AIMS scores, measured at 20-minute intervals. Data from the separate experimental session testing IRL790 (20 mg/kg) are represented with dashed lines. IRL790 resulted in a dose-dependent suppression of dyskinesia which was statistically significant at 10 mg/kg and 20 mg/kg ($p < 0.01$, and $p < 0.0001$, respectively; repeated measures ANOVA followed by Dunnett's test, confirmed with Mann-Whitney U-test, based on total composite scores, $p < 0.05$ and $p < 0.01$ at 10 and 20 mg/kg, respectively).

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Figure 6.

Effects of IRL790 on rotations in AIMS model, external study.

Rats were subjected to unilateral 6-OHDA lesion followed by four weeks treatment with L-DOPA, 6.5 mg/kg dose s.c. (plus benserazide 12 mg/kg), in order to establish full expression of dyskinesias. On the test day IRL790 was administered at 1, 3 and 10 mg/kg μ mol/kg s.c. 20' before L-DOPA (6.5 mg/kg s.c.) (n=8). Controls received L-DOPA only (n=8). In a separate experimental session (lower panel), L-DOPA+IRL790, 20 mg/kg, was tested (n=7/group). All groups were balanced to have similar AIMS scores at baseline. Shown are means \pm SEM of rotations, measured at 20-minute intervals, data from first experimental session testing 1-10 mg/kg in the upper panel, data from the experimental session testing IRL790 20 mg/kg in the lower panel. IRL790 did not display any reduction of the rotational response to L-DOPA.

Figure 7.

Dose response analysis of total adverse involuntary movements (AIMs) score.

Graph based on data from external study (IRL790 1, 3, 10 and 20 mg/kg, and controls). Controls are drawn at log (dose) = -2. N= 7-8/dose. Shown are means \pm SEM. ED50 = 4 mg/kg (black circle) was estimated by means of fitting a sigmoidal curve (Hill equation), assuming Emin=50 (no reduction), Emax 25 (50% reduction of AIMs).

Figure 8.

Effects of IRL790 on *ex vivo* monoaminergic neurochemistry.

Analyte is denoted by: NA = norepinephrine, NM = normetanephrine, DO = 3,4-dihydroxyphenylacetic acid (DOPAC), DA = dopamine, HIA = 5-hydroxyindoleacetic acid (5-

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HIAA), HV = homovanillic acid (HVA), MT = 3-methoxytyramine (3-MT), HT = 5-hydroxytryptamine (5-HT). Brain region: L = limbic region, S = striatum, C = cortex. Brains were removed and dissected 60' after administration of IRL790 (3.7, 11, 33 or 100 $\mu\text{mol/kg}$, s.c.). Controls received saline. N=4. Shown are means \pm SEM, expressed as percentage of control group mean. * $p<0.05$, ** $p<0.01$. $p<0.001$ vs control group, Students t-test.

Figure 9.

Effects of IRL790 on rat brain regional dialysate levels of monoamines.

Dialysate levels of DA and NA in the prefrontal cortex (upper panel) and striatum (lower panel) were monitored 0-180 minutes following administration of IRL790 (16 $\mu\text{mol/kg}$, light blue, 50 $\mu\text{mol/kg}$, dark blue) Shown are the mean dialysate levels/20-min sample \pm SEM, expressed as percentage of baseline values (average of the 3 fractions collected 40 to 0 minutes before dosing). N= 5-7/group (NA Striatum, 16 $\mu\text{mol/kg}$, n=2). Significant differences between effect of vehicle and IRL790 at specific time-points are indicated as follows: * $p<0.05$ ** $p<0.01$ *** $p<0.001$ (repeated measures ANOVA, followed by Fischer's LSD post-hoc test)

Figure 10.

Effects of IRL790 on rat brain regional dialysate levels of ACh.

Dialysate levels of ACh in the prefrontal cortex (upper panel) and ventral hippocampus (lower panel) were monitored 0-180 minutes following administration of IRL790, 50 $\mu\text{mol/kg}$, or vehicle (saline). Shown are the mean dialysate levels/20-min sample \pm SEM, expressed as percentage of baseline values (average of the 4 fractions collected 60 to 0 minutes before dosing). N= 6 /group, pfc, 3-4/group, ventral hippocampus. Significant differences between effect of vehicle and IRL790 at specific time-points are indicated as follows: * $p<0.05$ ** $p<0.01$ *** $p<0.001$ (repeated measures ANOVA, followed by Fischer's LSD post-hoc test)

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Figure 11.

Effects of IRL790 on expression of immediate early genes (*IEGs*).

Brains were removed and dissected 65' after administration of IRL790 at 11, 33 and 100 $\mu\text{mol/kg}$, s.c. Controls received saline. Brain region: L = limbic region, S = striatum, F = frontal cortex. Arc (activity-related cytoskeletal protein), c-Fos (cellular DNA-binding proteins encoded by the c-Fos genes), EGR1 (early growth response protein 1), Homer1 (a postsynaptic density adaptor protein), Npas4 (neuronal PAS domain protein 4), and Nptx2 (neuronal pentraxin-2 protein) were measured by qPCR. Results are presented as percentage of controls (means \pm SEM; n=5 animals/treatment group). Statistics: Student's t-test (2-tailed), * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. controls. Data is sorted by gene, then by region and then by ascending dose from left to right.

Figure 12.

Gene expression map on IRL790 and selected compounds implicated in PD.

Shown are w^*c loadings, component 1 vs component 2, from a PLS regression model of effects on IEGs (independent variable block) vs. treatment with different test compounds (dependent variable block of discriminant variables denoting treatment). Loadings indicate correlation patterns among variables (w^* loadings), and effect patterns and clusters among compounds (c loadings). Compounds located close to each other share major effect patterns, while diametrically opposing positions reflect opposing effects. Variable loadings indicate effects governing mapping of compounds. Variables located close to each other tend to be correlated, while diametrically opposing positions reflect negative correlations. Variables located in orthogonal directions are uncorrelated.

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Figure 13.

IRL790 docked into the binding site of the dopamine D3 receptor crystal structure (pdb entry 3pbl).

A ribbon representation of the receptor with three key residues important for agonist binding and the disulfide bridge between ECL2 and TM3 are shown. The shape of the binding site is illustrated by the accessible surface for the parts of the protein that are in close proximity to the ligand, represented by a white mesh showing how close an oxygen atom in a water molecule could be the protein surface without any steric repulsion.

Figure 14.

An overlay of the binding sites of the dopamine D3 receptor crystal structure (dark green) and the homology model of the dopamine D2 receptor (light green).

A ribbon representation of the receptor with the only residue position in ECL2 (C+1) with a mutation in the binding site (Ile in D2 and Ser in D3) are shown with thick bond representation. Other key residues (Asp^{3.32} in TM3 and Ser^{5.42} and Ser^{5.43} in TM5) are shown with thin bond representations. The difference in shape of the binding site are illustrated by the accessible surface for the parts of the protein that are in close proximity to the ligand (D3 with a mesh and D2 with a solid surface representation).

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Tables

Table 1 IRL790 IC50/Ki values for selected targets.

| Assay | Radioligand (type) | Source | IC50 (μM) | Ki (μM) | Reference compound |
|-----------------------------------|---|---------|---------------------------|-------------------------|-----------------------|
| D3 (h) | [^3H]Me-spiperone (antagonist) | CHO | 0.41 | 0.09 | (+)butaclamol |
| D2S (h) | [^3H]Me-spiperone (antagonist) | HEK-293 | 1.6 | 0.54 | (+)butaclamol |
| D2S (h) | [^3H]7-OH-DPAT (agonist) | HEK-293 | 1.9 | 0.75 | 7-OH-DPAT |
| Sigma 1 (h) | [^3H](+)pentazocine (agonist) | Jurkat | 1.7 | 0.87 | haloperidol |
| 5-HT1A (h) | [^3H]8-OH-DPAT (agonist) | HEK-293 | 3.6 | 2.2 | 8-OH-DPAT |
| 5-HT2A (h) | [^{125}I](\pm)DOI (agonist) | HEK-293 | 9.9 | 7.4 | (\pm) DOI |
| norepinephrine transporter (h) | [^3H]nisoxetine (inhibitor) | CHO | 11 | 8 | protriptyline |
| 5-HT7 (h) | [^3H]LSD (agonist) | CHO | 29 | 11 | serotonin |
| α 2C (h) | [^3H]RX 821002 (antagonist) | CHO | 45 | 15 | yohimbine |

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| | | | | | |
|---------------------------------|---|------------------------|------|---|--------------------------------|
| α 1 (non-selective; rat) | [³ H]prazosin (antagonist) | Rat cerebral cortex | >100 | - | prazosin |
| α 2A (h) | [³ H]RX 821002 (antagonist) | CHO | >100 | - | yohimbine |
| dopamine transporter (h) | [³ H]BTCP (antagonist) | | >100 | - | BTCP |
| H3 (h) | [³ H]N- α -Me- histamine (agonist) | CHO | >100 | - | R(α)-Me- histamine |
| MAO-A (rat) | [³ H]Ro 41-1049 (inhibitor) | Rat cerebral cortex | >100 | - | clorgyline |
| MAO-B (rat) | [³ H]Ro 19-6327 (inhibitor) | Rat cerebral cortex | >100 | - | (R)-deprenyl |

The abbreviation (h) denotes human recombinant protein

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Table 2 Agonist effects in cellular functional assays (human receptors)

| Assay | EC50 | Source | Measured component | Reference |
|--------------|--------------------------|---------------|---------------------------|------------------|
| D2S | n.c.* | HEK-293 cells | Impedance | SOP 1C531 |
| D3 | n.c. | CHO cells | cAMP | SOP 1C374 |
| 5-HT1A | $> 1.0 \times 10^{-4}$ M | HEK-293 cells | Impedance | SOP 1C913 |
| 5-HT2A | n.c. | HEK-293 cells | IP1 | SOP 1C865 |

* n.c. EC50 not calculable due to lack of effect. SOP refers to standard operating procedures used by CEREP.

Table 3 Antagonist effects in cellular functional assays (human receptors)

| Assay | IC ₅₀ | K _B | Stimulus | Source | Measured component | Reference |
|--------|----------------------------------|----------------|---------------------------|----------------------|--------------------|-----------|
| D2S | 13 μ M | 0.87 μ M | Dopamine (30 nM) | HEK- 293 cells | Impedance | SOP 1C531 |
| D3 | 9.8 μ M | 2.3 μ M | Dopamine (10 nM) | CHO cells | cAMP | SOP 1C374 |
| 5-HT1A | n.c. due to agonist effect | | 8-OH- DPAT (100 nM) | HEK- 293 cells | Impedance | SOP 1C913 |
| 5-HT2A | n.c. | | Serotonin (100 nM) | HEK- 293 cells | IP ₁ | SOP 1C865 |

* n.c. IC₅₀ not calculable due to lack of effect. SOP refers to standard operating procedures used by CEREP.

Figures

Figure 1

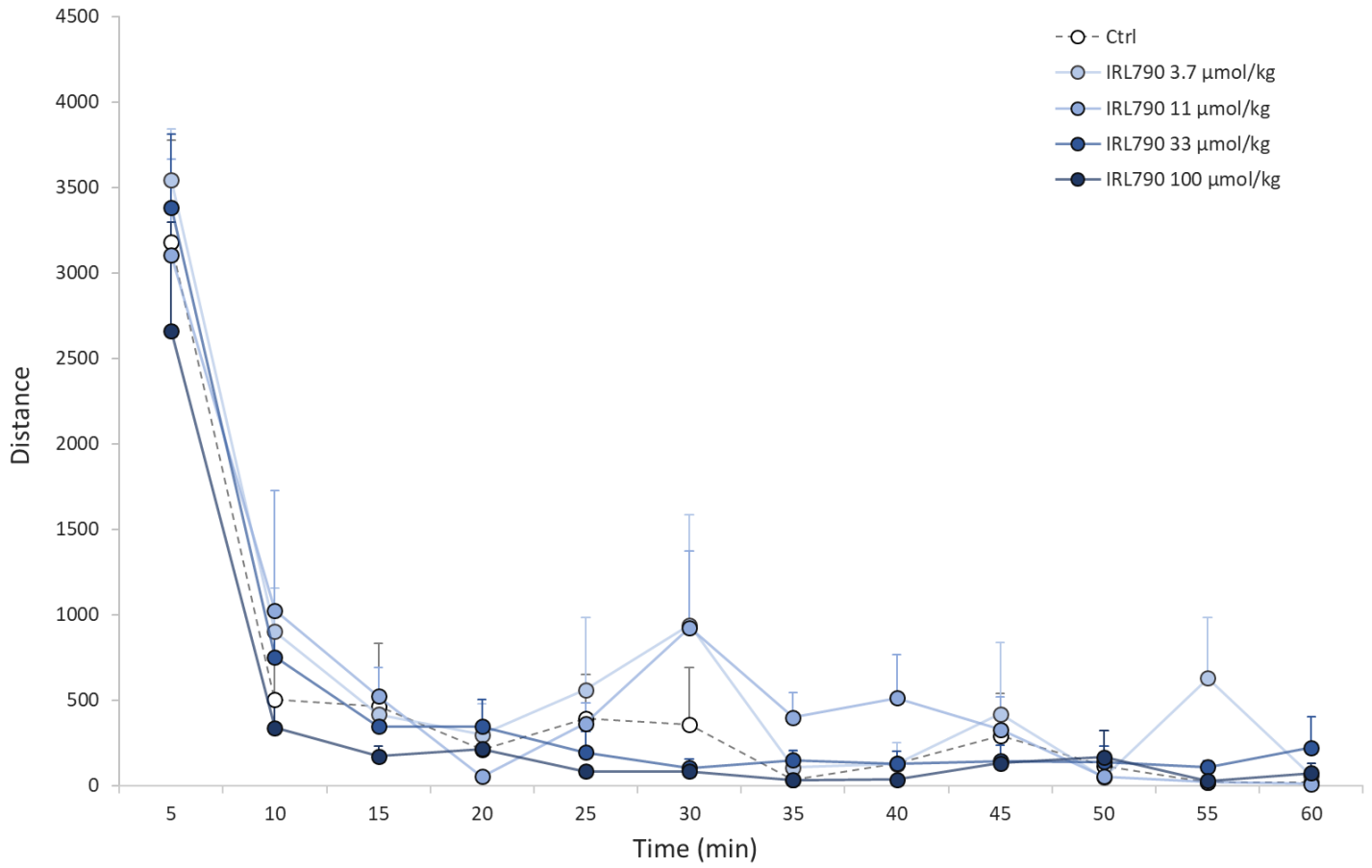


Figure 2

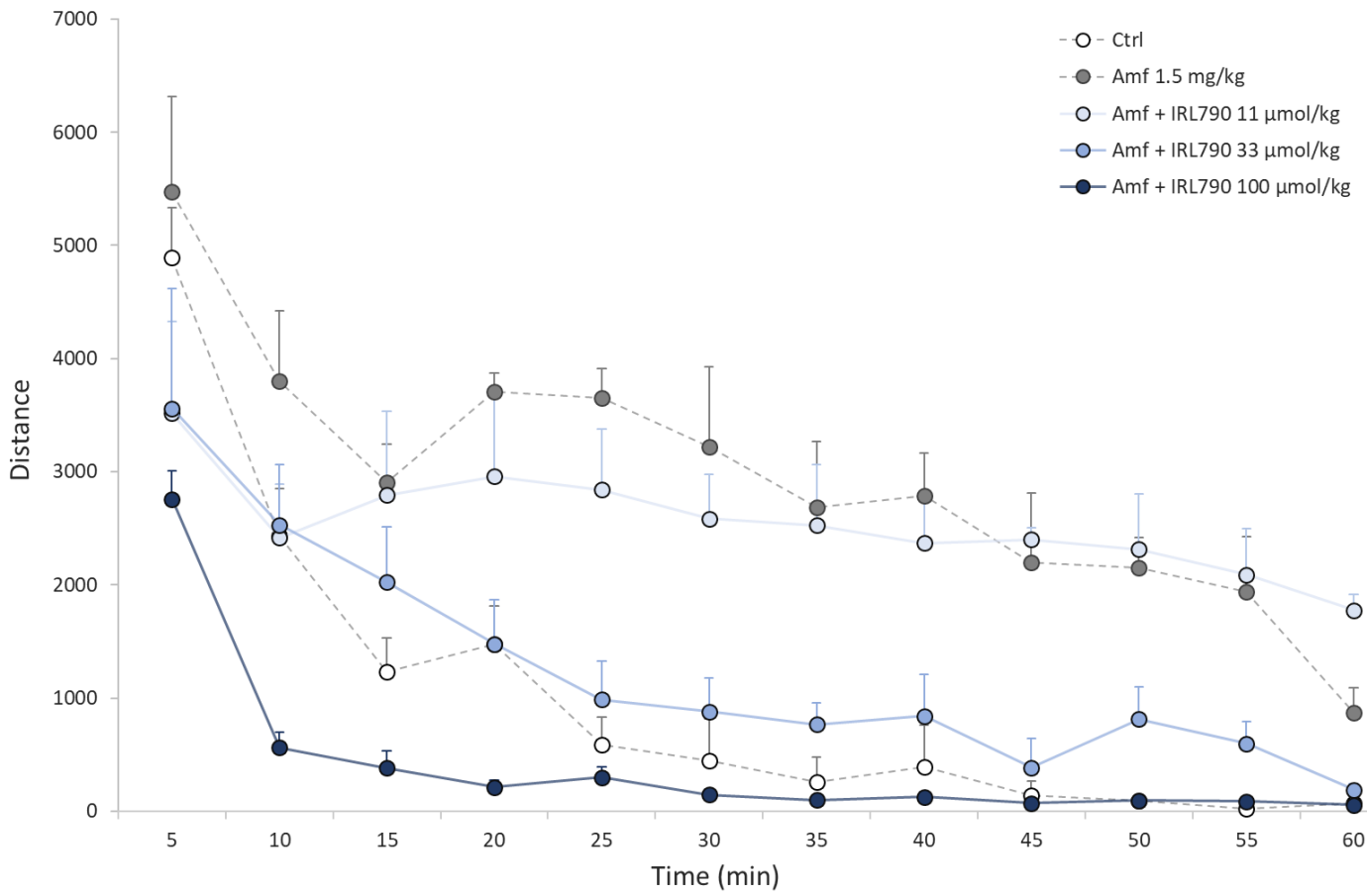


Figure 3

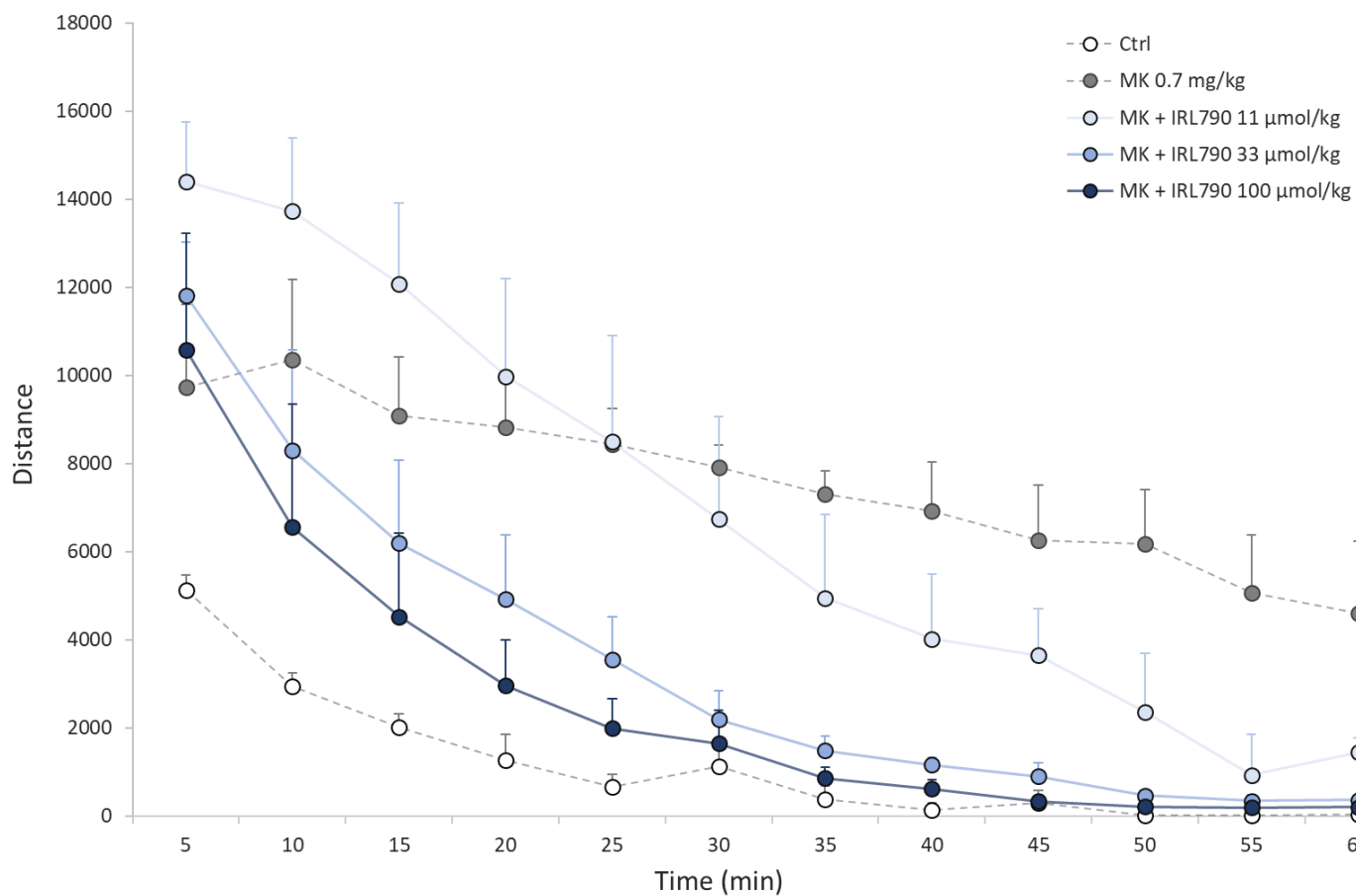


Figure 4

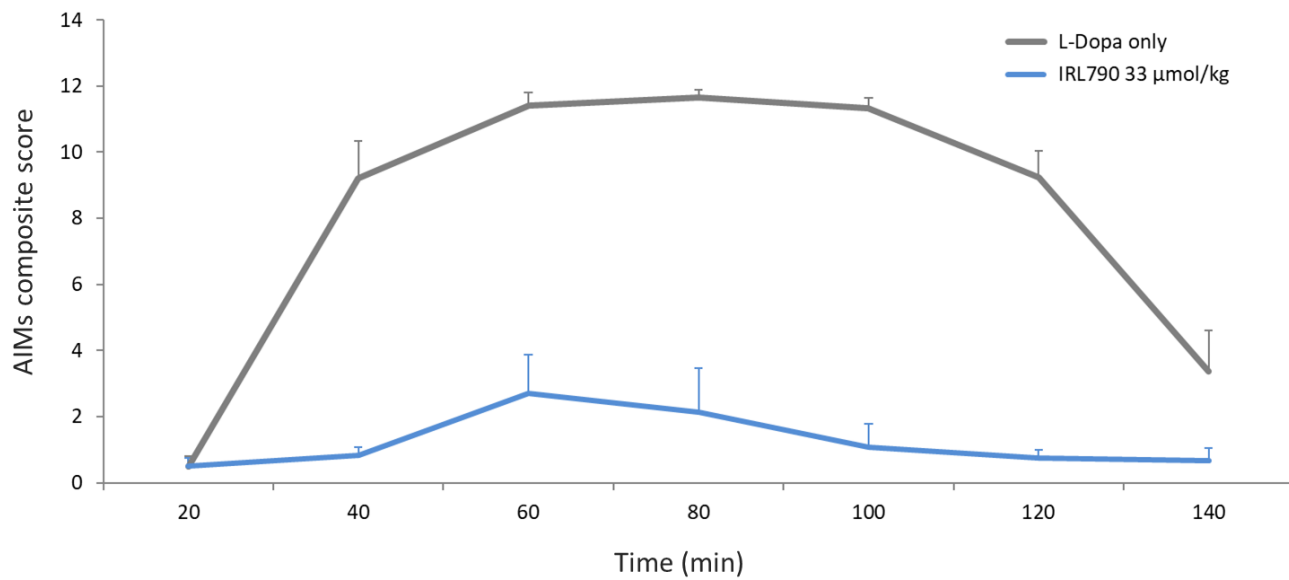


Figure 5

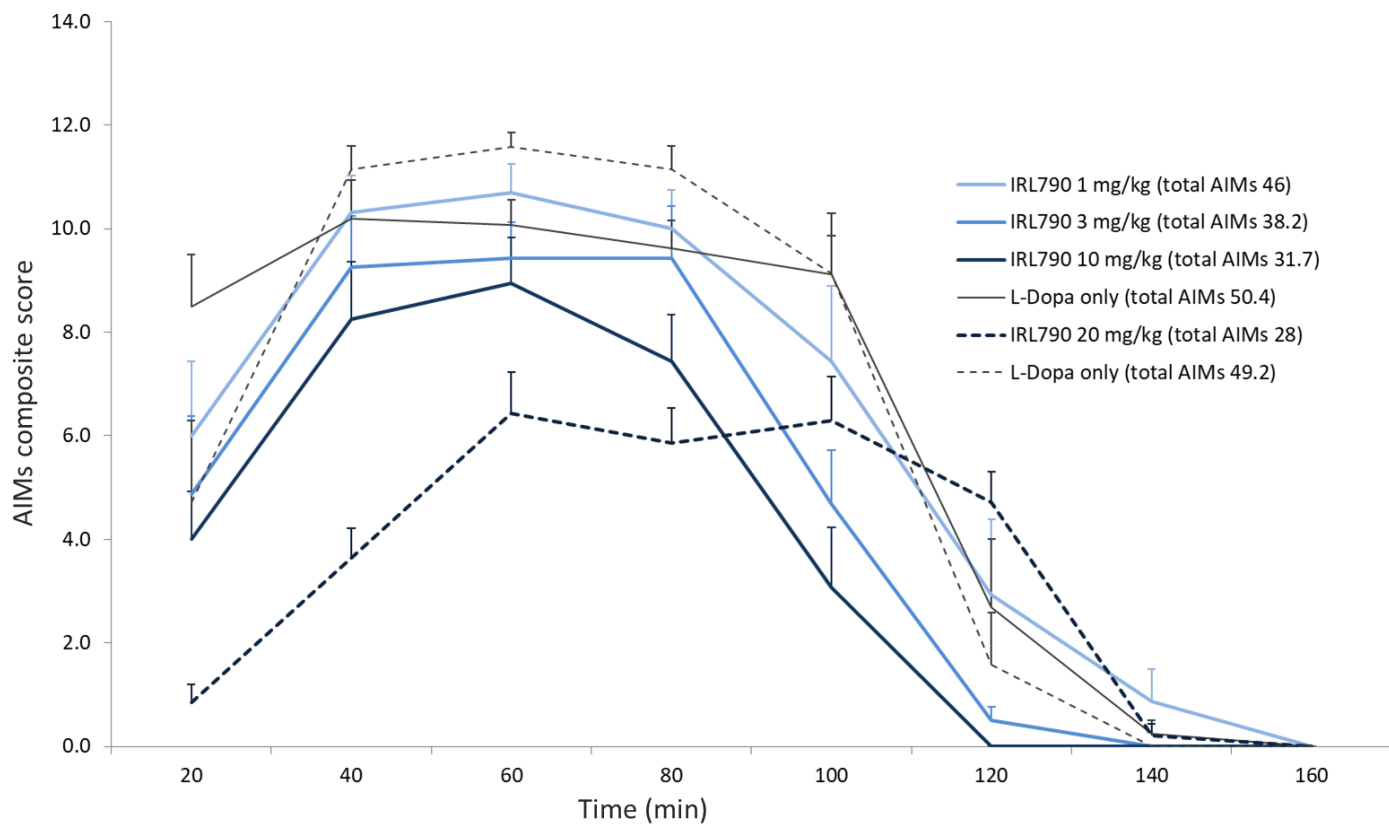


Figure 6

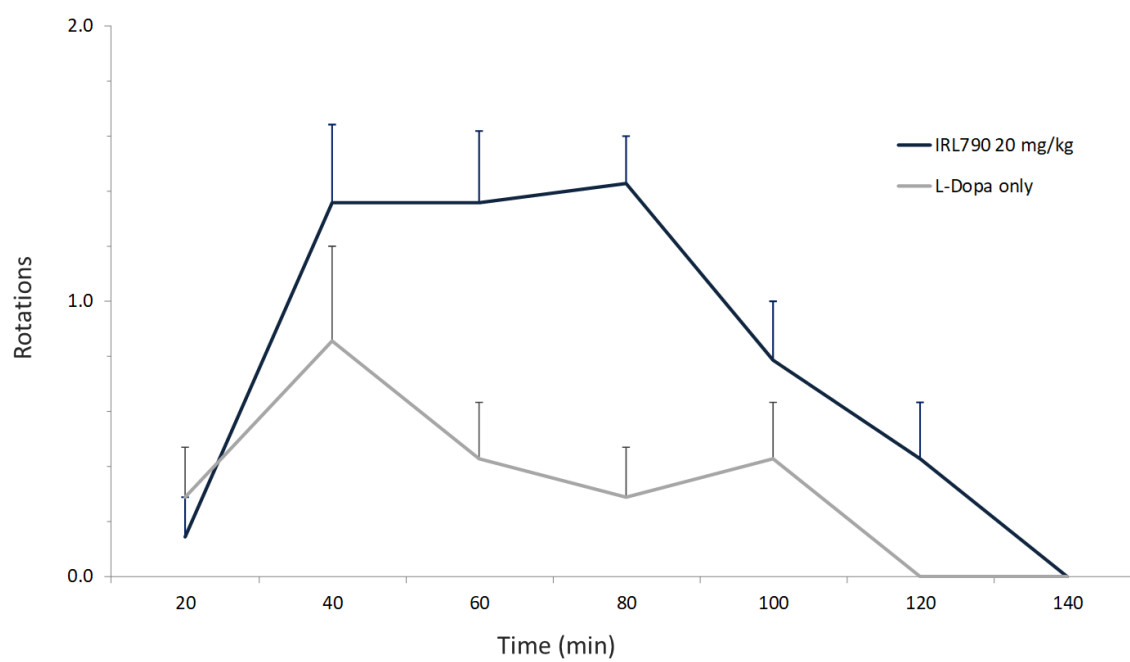
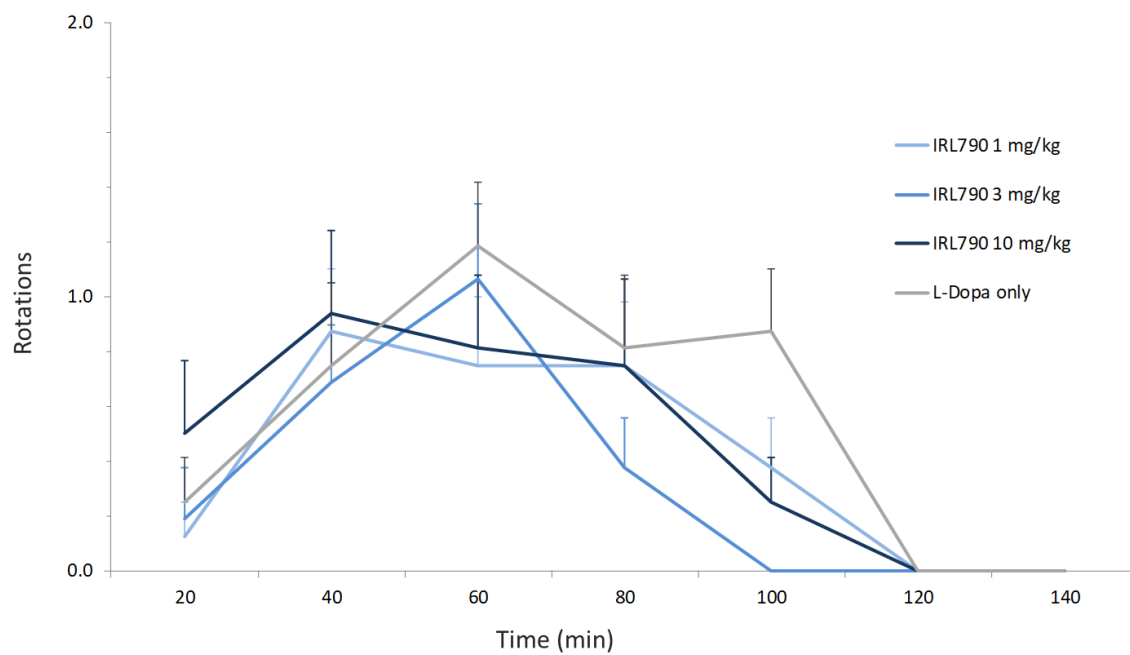


Figure 7

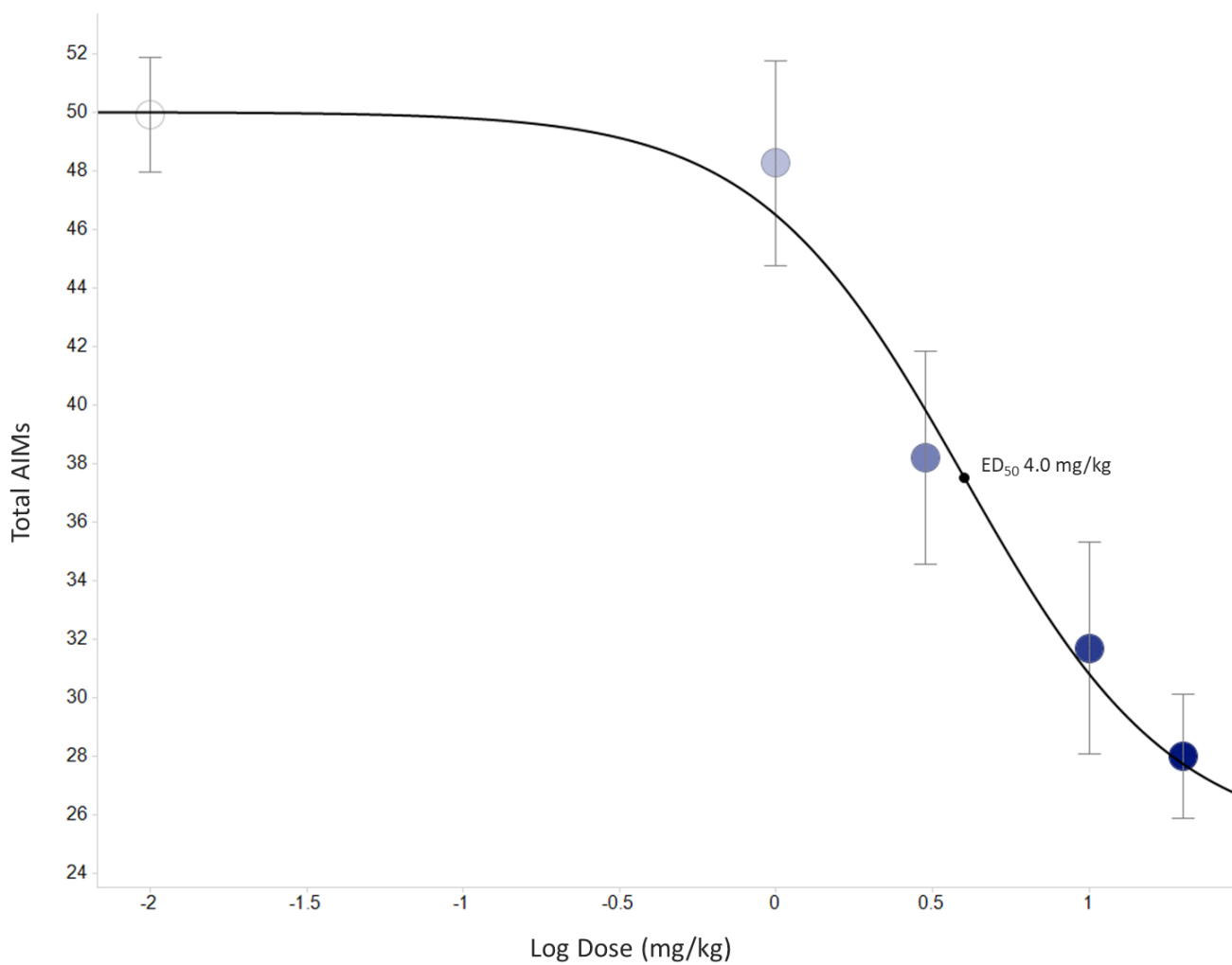


Figure 8

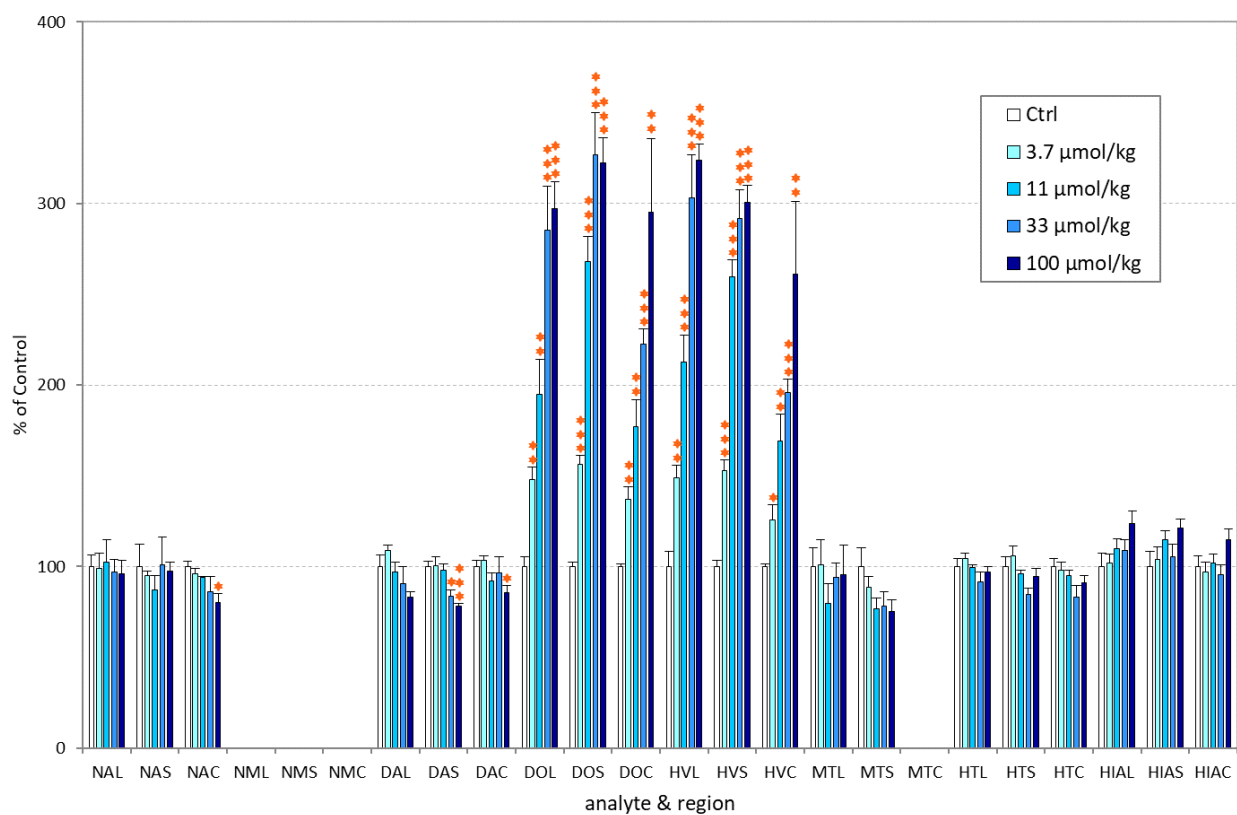


Figure 9

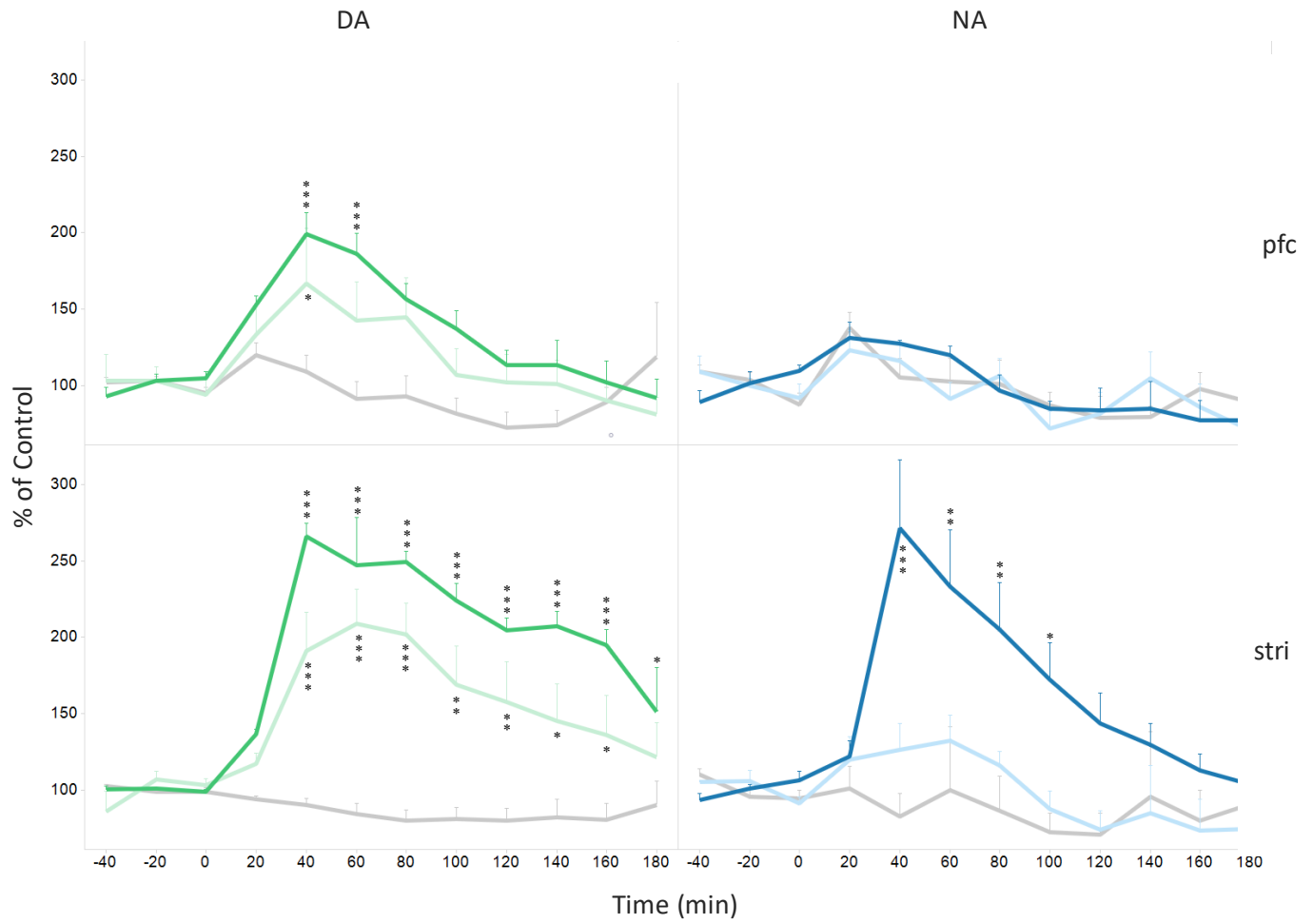


Figure 10

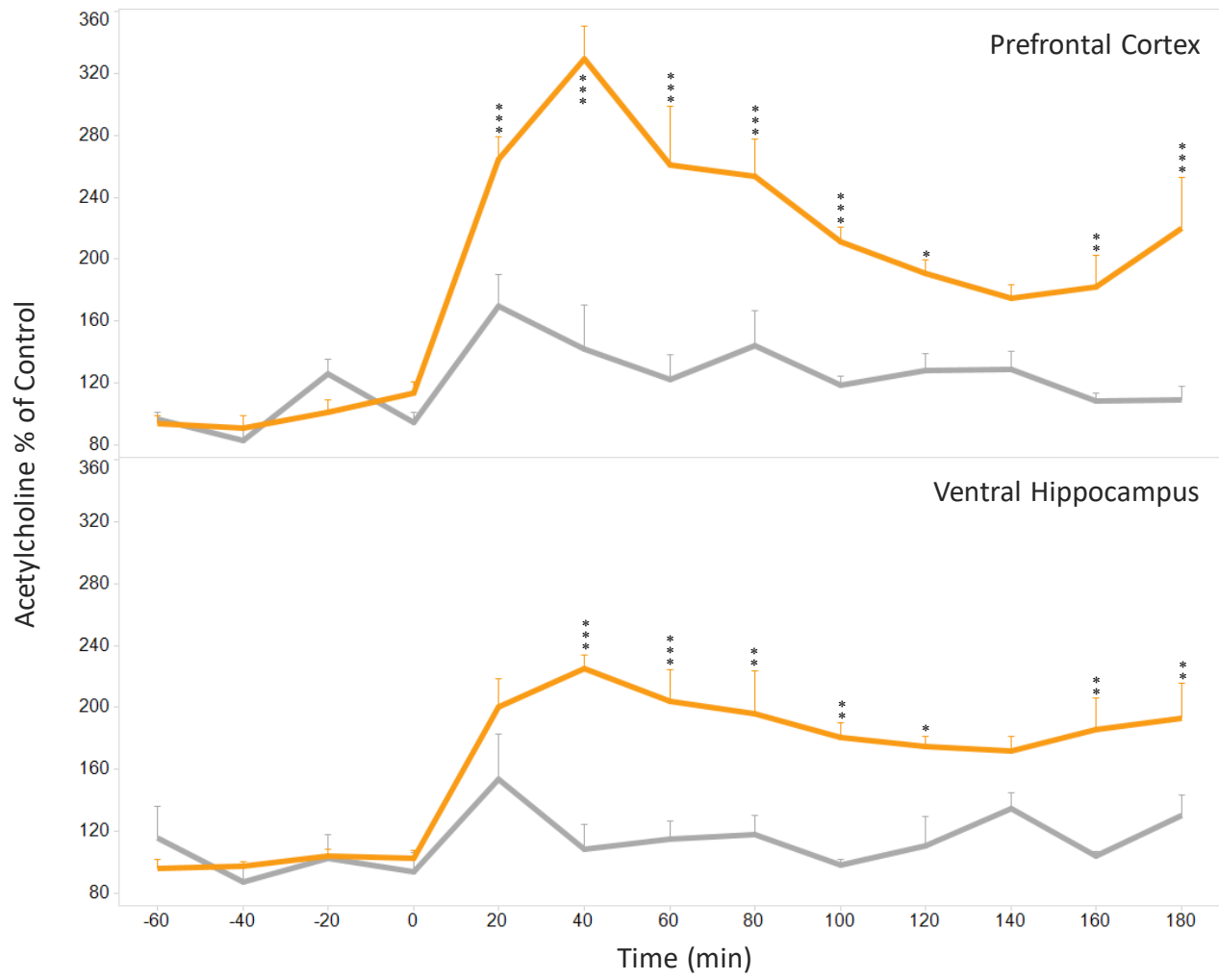


Figure 11

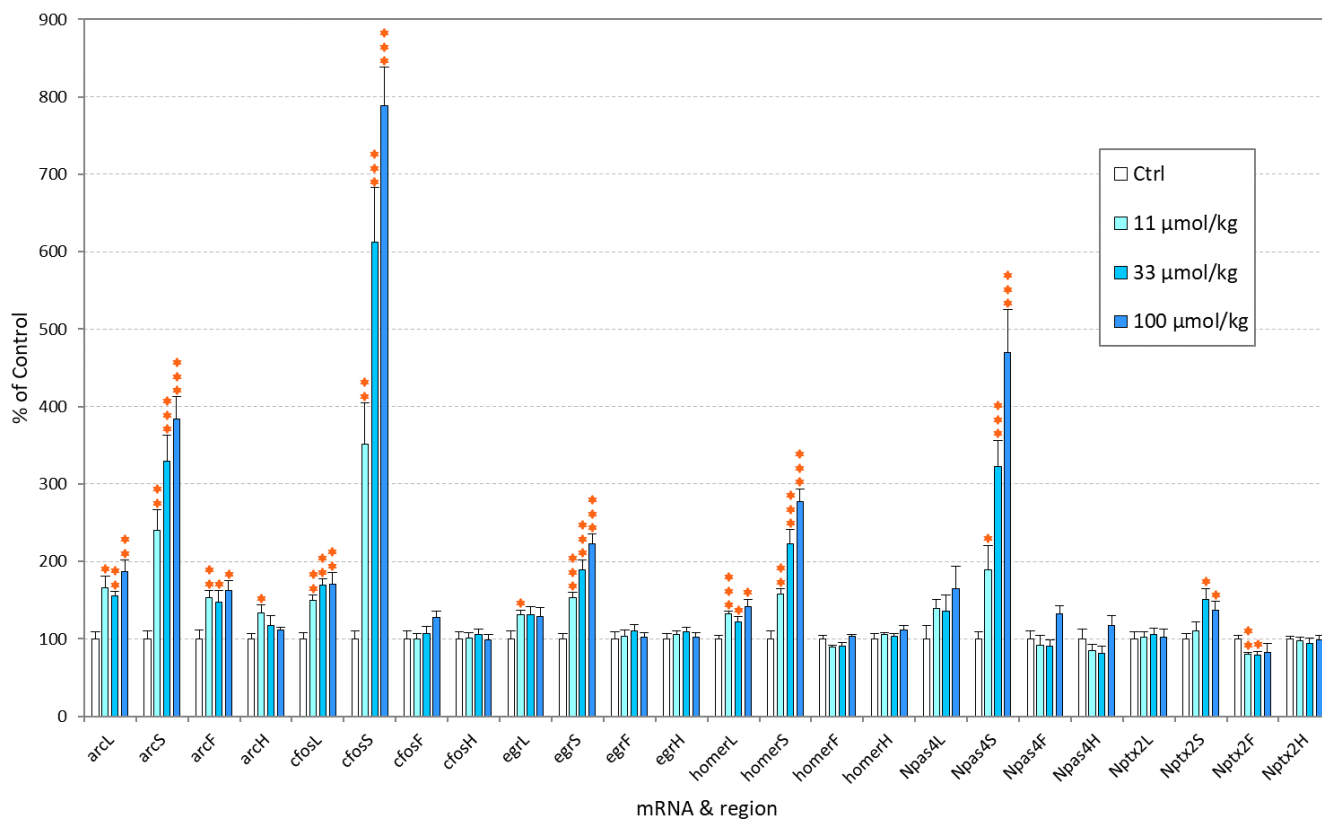


Figure 12

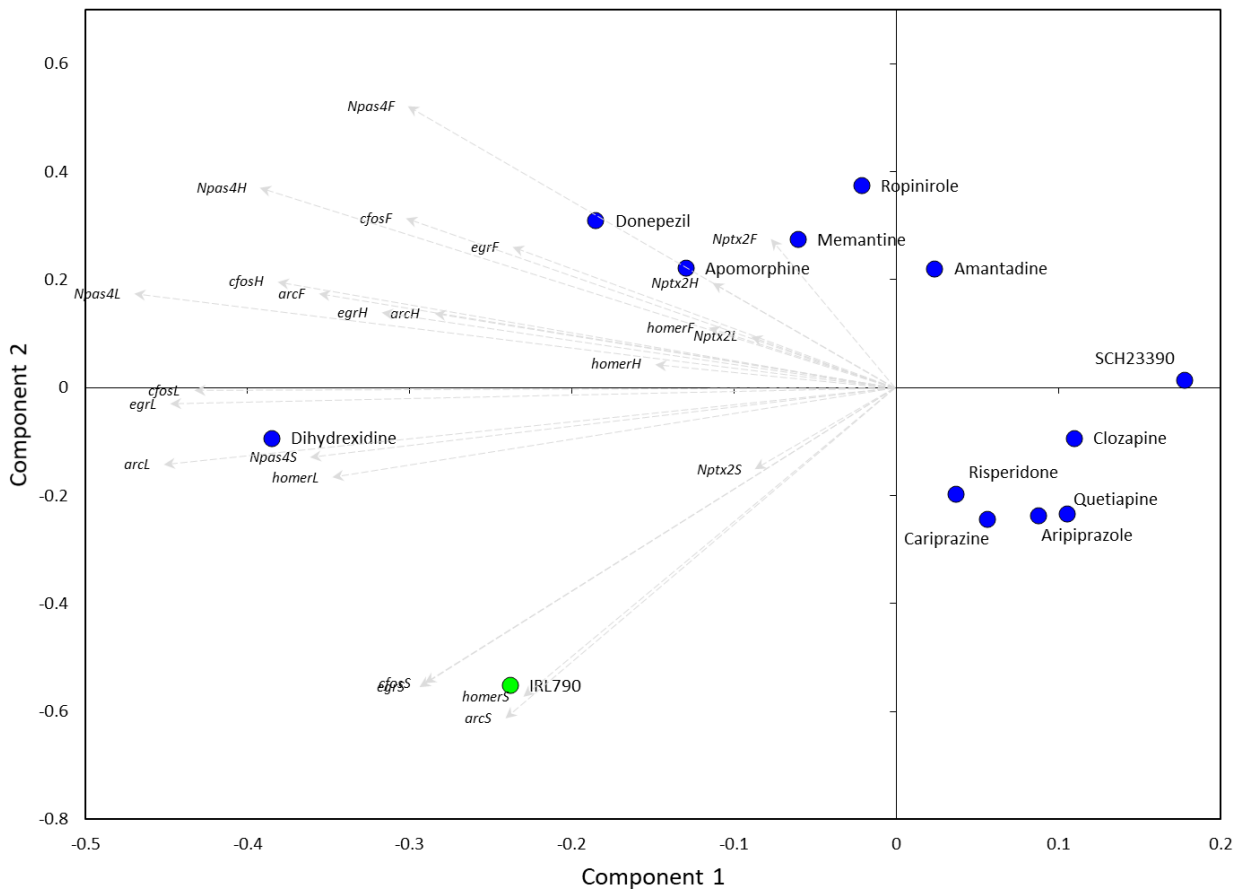


Figure 13

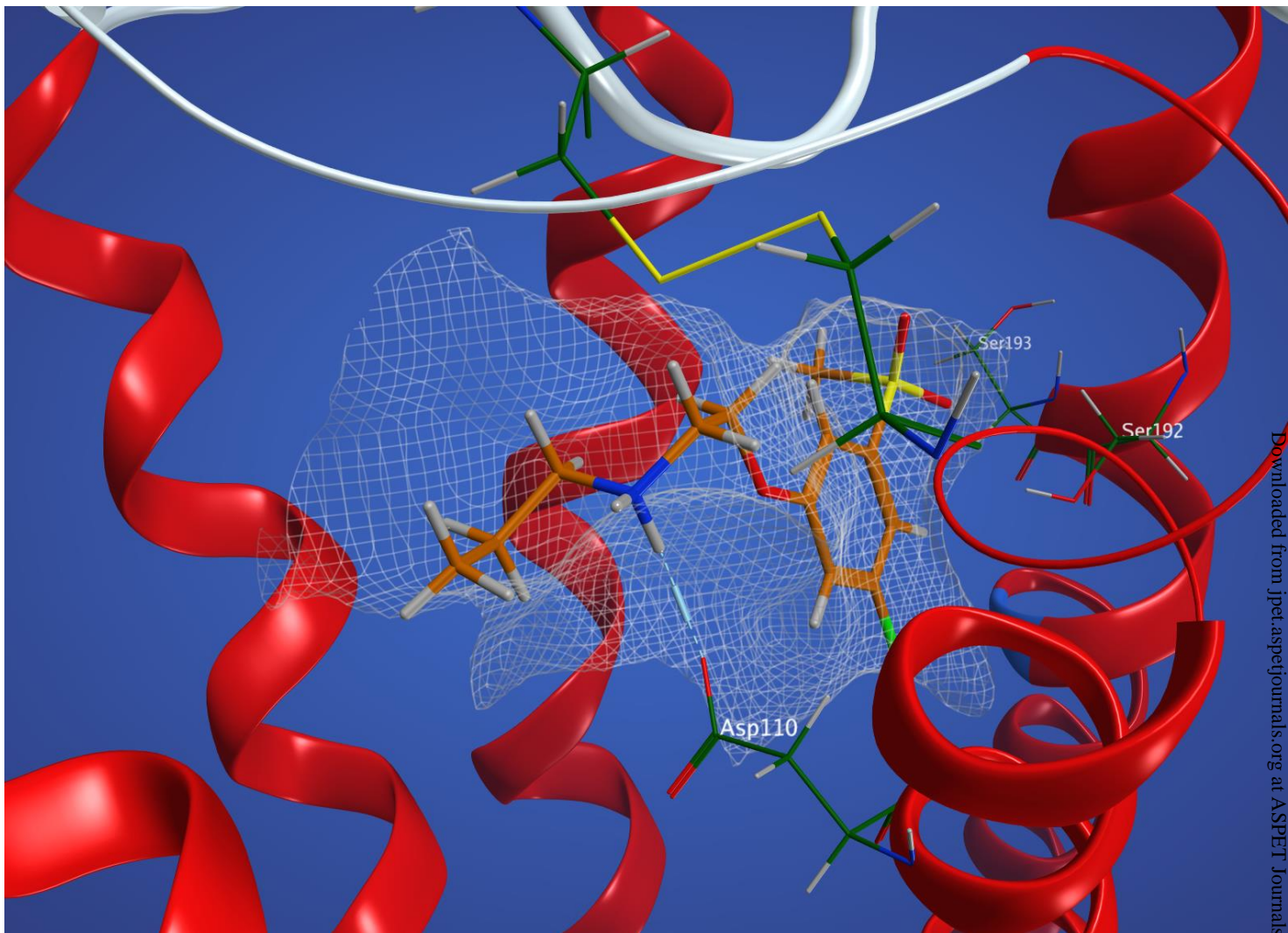
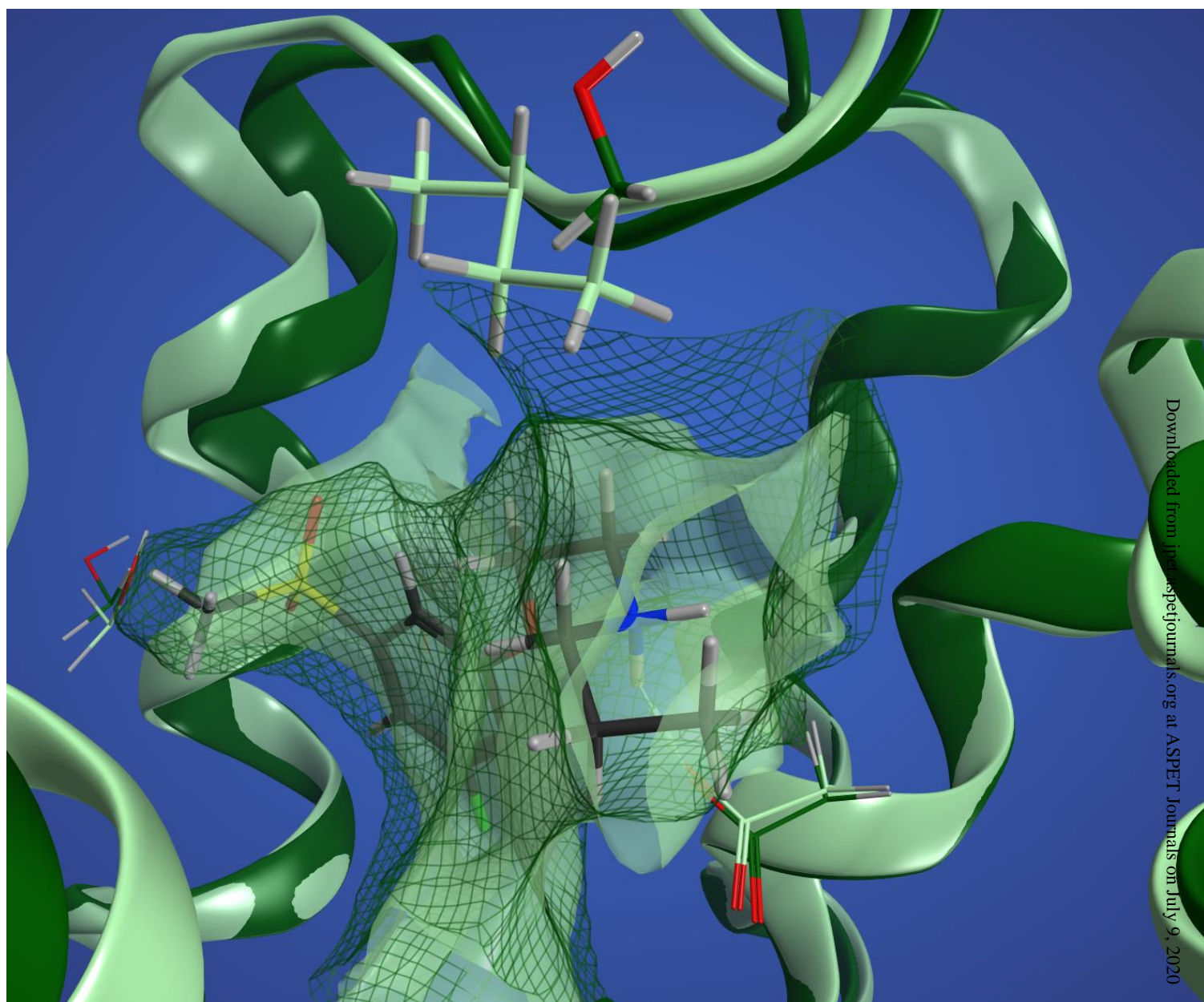


Figure 14



Footnotes

- a) This work was funded by Integrative Research Laboratories Sweden AB, Sweden
- b) Part of this work was presented at the Dopamine 2016 conference, Vienna, Austria, Sept 5-9 2016. Waters, N., et al. Pharmacology of IRL790, a psychomotor stabilizer for the treatment of L-dopa induced dyskinesias and psychosis in Parkinson's disease. Poster (We-66C)
- c) Reprint requests should be addressed to Susanna Waters, Integrative Research Laboratories Sweden AB, A Wallgrens Backe 20, SE 413 46 Göteborg, Sweden.
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